Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Affinity Separation of Trypsin from Goat Pancreatic Extract Using a Polyethersulfone Ultrafiltration Membrane

Sumana Mukherjee[a]; Debashis Roy[a]; Pinaki Bhattacharya[a]
[a] Chemical Engineering Department, Jadavpur University, Kolkata, India

Online publication date: 22 June 2010

Separation Science and Technology, 43: 3333–3350, 2008

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390802215057

Taylor & Francis
Taylor & Francis Group

Affinity Separation of Trypsin from Goat Pancreatic Extract Using a Polyethersulfone Ultrafiltration Membrane

Sumana Mukherjee, Debashis Roy, and Pinaki Bhattacharya
Chemical Engineering Department, Jadavpur University, Kolkata, India

Abstract: Ultrafiltration of goat pancreatic extract to obtain trypsin, using Soybean Trypsin Inhibitor as affinity ligand and a 30 kDa MWCO polyethersulfone membrane, is studied by examining the effect of varying transmembrane pressure and pancreatic extract: wash buffer ratio on active trypsin yield and flux/throughput profiles. For all process conditions considered, no detectable trypsin activity is found in the washing-phase permeates confirming excellent ligand-trypsin binding. Maximum trypsin yield obtained, at 1:1 feed ratio and 4 kg/cm^2 pressure, is 74%. Mass flux of eluted protein as well as of washed-off impurities are also maximized under these conditions.

Keywords: Affinity separation, goat pancreatic extract, polyethersulfone ultrafiltration membrane, trypsin

INTRODUCTION

Trypsin is a serine protease with substrate specificity based upon positively charged lysine and arginine side chains (1), which is naturally produced by the pancreas. It was the first enzyme to be isolated in sufficiently pure form for chemical and enzymological studies. Due to its powerful proteolytic function and specificity of action, trypsin has a wide range of applications e.g. as a critical intermediary in the manufacture of insulin, in cell culture applications, in the production of recombinant proteins for clinical uses, as a debriding agent in wound care, as an oral

Received 8 October 2007; accepted 6 March 2008.
Address correspondence to Debashis Roy, Chemical Engineering Department, Jadavpur University, Kolkata 700032, India. Telefax: +91-33-24146378. E-mail: deebie_roy@yahoo.com

treatment for inflammatory edema, hematoma and pain associated with a wide range of external and internal wounds, in vaccine production, as a digestive enzyme etc. As per 2004 estimates, a five-fold increase in global demand for trypsin is predicted in the next five years (2).

Isolation of trypsin from animal pancreas has been reported from many sources e.g. bovine (3), porcine (4,5), ovine (5,6), goat (7), moose (8), whale (9), elephant seal (10), African lungfish (11), swine (12,13), rat (14), turkey (15,16), and human (17,18). Studies (4–6,15–16,19) suggest that mammalian trypsins show a marked similarity in their overall physical and chemical properties although their amino acid compositions may still differ significantly. Sinha and Das (20), who reported the isolation of trypsin from goat pancreas and its crystallization, found goat trypsin to resemble bovine trypsin in a number of properties e.g. esterase activity, pH optimum, reactivity with inhibitors and sedimentation coefficient, although goat trypsin was more stable than bovine trypsin. In a subsequent study (7) these authors noted significant differences in amino acid compositions of goat, bovine, ovine, porcine, and human trypsins although in case of goat and bovine trypsins the differences were less marked. The average molecular weight of goat trypsin was estimated (7) as ca. 22.6 kDa.

Affinity ultrafiltration is a two-step protein bioseparation process consisting of the "washing" and "elution" phases. In the "washing" phase, the target enzyme binds selectively with a macromolecular affinity ligand forming a ligand-enzyme complex which is trapped (retained) by the membrane having molecular weight cut-off (MWCO) less than the molecular weight of the ligand-enzyme complex (but greater than that of the unbound target enzyme) whereas all undesirable molecules (impurities) that do not bind to the ligand, freely permeate the membrane and are "washed off" from the system by the wash buffer. In the "elution" phase, the target enzyme dissociates from the ligand-enzyme complex by addition of an appropriately selected "eluting agent" (e.g. a pH buffer, that changes the medium pH facilitating dissociation of the target enzyme from the ligand) and the eluant containing the target enzyme passes through the membrane while the macroligand is retained, which may be reused after regeneration.

Some of the advantages of affinity ultrafiltration over other protein purification techniques include commercial availability of a wide range of high-throughput ultrafiltration systems and of affinity macroligands that facilitate rapid separation of large feed volumes, repeated use of equipment enabling consecutive purification of different proteins and simple scale-up and automation procedures. In particular, as far as affinity-based protein separation techniques are concerned, affinity ultrafiltration scores over affinity chromatography on several counts as noted below. The soluble macroligands used in affinity ultrafiltration require no

spacer molecules that are unavoidable with insoluble matrices employed in affinity chromatography, thus the synthesis of the former is a simple, low-cost process. Again, a soluble ligand is much less likely to be prone to degradation by attrition or compression in contrast to conventional affinity chromatography where this vulnerability is a real threat for affinity matrices tightly packed into a column which can easily plug and foul. Further, an instantaneous binding between the ligand and the enzyme as in affinity ultrafiltration essentially translates into high throughput. Besides, scaleup of a chromatographic separation process is usually very complicated and may present unforeseen challenges as opposed to the remarkable ease of scaleup of an ultrafiltration process.

Probably the earliest experimental study on affinity ultrafiltration was reported by Bartling and Barker (21) who separated a mixture of trypsin and peroxidase using Soybean Trypsin Inhibitor (STI) cross-linked with Woodward's reagent K, as the affinity macroligand. Adamski-Medda et al. (22) reported the ultrafiltration of a trypsin-chymotrypsin mixture using dextran-p-aminobenzamidine as the ligand. Affinity ultrafiltration studies of trypsin-chymotrypsin mixtures, using different ligands, have been subsequently reported by Choe et al. (23) (using STI-dextran polymer); by Luong et al. (24) (using N-acryloyl-m-aminobenzamidine) and by Vedajnananda et al. (25) (using STI, cross-linked with Woodward's reagent-K). Male et al. (26) employed affinity ultrafiltration to isolate urokinase from its artificial mixture with peroxidase as well as from crude urine using an affinity copolymer of N-acryloyl-m-aminobenzamidine and acrylamide. Glatz and Novak (27) examined the affinity ultrafiltration of egg white lysozyme. Using BSA as a stereoselective affinity macroligand, Romero and Zydney (28–31) employed a multi-stage tangential flow affinity ultrafiltration process to separate enantiometric mixtures of d- and l-tryptophan, and study its various aspects. Rao and Zydney (32) investigated the affinity ultrafiltration of BSA and ovalbumin with Cibacron Blue as the ligand.

There are few reports on affinity ultrafiltration of pancreatic trypsin. In their seminal study, Luong et al. (33) successfully isolated trypsin from crude porcine pancreatic extract using a novel, multistage, continuous affinity ultrafiltration process with provision for recirculation of the eluant as well as the affinity macroligand. They used membranes with MWCO of 10^4 and 10^5, a water-soluble, high molecular weight ($>10^6$) polymer incorporating N-acryloyl-m-aminobenzamidine as the affinity macroligand, 50 mM Tris (pH 8) – 10 mM $CaCl_2$ as wash buffer and benzamidine as eluant.

Polyethersulfone membranes have been widely used in ultrafiltration studies. Lebrun et al. (34) used a 10 kDa modified polyethersulfone membrane for ultrafiltration of a bovine haemoglobin peptidic hydrolysate.

Cowan and Ritchie (35) used a polyethersulfone membrane chemically modified by polymerization of styrene in the membrane pores for ultrafiltration of whey proteins α-lactalbumin (14 kDa) and β-lactoglobulin (18 kDa), and obtained a five-fold enhancement in selectivity of the modified membrane relative to that of the raw membrane. Cheang and Zydney (36) also examined the ultrafiltration of α-lactalbumin and β-lactoglobulin using 30 kDa cellulosic as well as polyethersulfone membranes. Croue et al. (37) investigated the membrane flux decline during ultrafiltration of NOM (Natural Organic Matters) fractions isolated from surface waters using an unstirred, dead-ended ultrafiltration cell equipped with flat-sheet, polyethersulfone membranes. Priyananda and Chen (38) investigated the ultrafiltration of mixtures of protein (BSA) and fatty acid salt (sodium caprate) with a 30 kDa polyethersulfone membrane at 50 kPa and 350 rpm in a stirred ultrafiltration cell. Boyd and Zydney (39) analyzed the effect of protein fouling on flux and retention characteristics of polyethersulfone ultrafiltration membranes, whereas Rahimpour et al. (40) reported the preparation of high performance, large sheet, asymmetric polyethersulfone ultrafiltration membranes. Polyethersulfone membranes are used for multiple clinical applications, particularly in nephrology (41) for the optimization of dialysis treatment. In this sphere, some of the areas of active interest include the kinetic study of albumin loss in pre-dilutional hemodiafiltration, cytokine removal using polyethersulfone membrane, evaluation of membrane performance and albumin loss in post-dilutional hemodiafiltration, and a study of the effect of glucose infusion on membrane permeability.

In the present study, affinity ultrafiltration is employed for isolation of trypsin from goat pancreatic extract using a setup quite similar to that of Makdissy et al. (37), i.e. an unstirred, dead-ended ultrafiltration cell equipped with flat-sheet polyethersulfone membranes. In particular, it is aimed to investigate the effect of process parameters, viz. feed composition and transmembrane pressure (TMP) on separation performance, i.e. resolution (determined as active trypsin yield) and throughput (evaluated in terms of volumetric/mass flux and permeated protein concentration profiles of the washing and elution phases), and identify, if possible, a set of process conditions that optimize performance.

EXPERIMENTAL

Materials

Membrane: A hydrophilic polyethersulfone membrane of 30 kDa MWCO [Omega^{TM}, PallGelman] was employed.

Ultrafiltration Module: An unstirred, dead-ended ultrafiltration module of volume 50 ml and effective filtration surface area of $10.18 \times 10^{-4}\,m^2$ was used.

Instruments: Pancreas homogenate was prepared in a homogenizer [OCI Instruments, OMNI Mixer 17106, 16000 rpm] placed in an ice beaker. Subsequent centrifugation of the homogenate was carried out in a cold centrifuge [Remi, C24] at 0°C. Permeate fractions were collected with fraction collector [Pharmacia Biotech., LKB Redi Fract]. For spectrophotometric studies, a programmable UV-visible spectrophotometer [Perkin Elmer, Lamda 25] was used. Ultrapure water was obtained using an ultrapure water system [Millipore Corp., MilliQ RG]. Besides, a BOD incubator-shaker [Indian Instruments, 0–60°C] and a digital pH meter [Systronics, MKVI] were also used.

Chemicals: Bovine serum albumin (BSA), Soybean Trypsin Inhibitor (STI) and Tosyl-L-arginine-methyl ester (TAME) were purchased from Sigma, N-ethyl-5-phenylisoxazolium-3-sulfonate (Woodward's Reagent K) from Fluka and the rest, viz. Tris (Hydroxymethyl amino-methane), Folin-Phenol reagent, sodium bicarbonate, potassium sodium tartrate, copper sulfate, sodium hydroxide, calcium chloride, potassium chloride, hydrochloric acid, and buffer tablets were all procured from Merck. All chemicals/reagents used were of analytical grade.

Methods

Analytical

Total protein concentration of the permeates of both the washing and the elution phases was determined spectrophotometrically at 600 nm following the method of Lowry et al. (42) using BSA as the standard. Esterolytic activity of trypsin was evaluated spectrophotometrically with Tosyl-L-arginine-methyl ester (TAME) in Tris-$CaCl_2$ buffer (pH 8.1) at 247 nm at 25°C following the method of Worthington (43). One unit of enzyme activity hydrolyses 1 μmole of TAME (p-toluene-sulfonyl-L-arginine methyl ester) per minute at 25°C, pH 8.1, in the presence of 0.01 M Ca^{++} ions.

Preparation of Goat Pancreatic Extract

Goat pancreas were collected from the gut wastes of a freshly slaughtered goat, immediately treated with 0.1 M Tris-HCl buffer (pH 8.3) and kept

in ice. It was cleaned (freed from mesentries, fat deposits, remnants of gut, arteries, veins etc.) then cut into very small pieces and dissolved in 50 ml 0.1 M Tris-HCl buffer (pH 8.3), and then homogenized at 5,000 rpm for 3 min at 0°C. The homogenate, which appeared as a viscous mass was dissolved by dissolving in 30 ml 0.1 M Tris-HCl buffer (pH 8.3) and filtered to remove solid debris and then centrifuged at 6500 rpm for 6 min. The supernatant was used as feed solution for trypsin isolation while the white pellets were rejected. Following earlier workers [e.g. Sinha and Das (20)] the feed solution (in 0.1 M Tris-HCl buffer) was activated at pH 8.3 in the presence of 0.1 M $CaCl_2$ and kept at 4°C. Total protein concentration of the feedstock thus obtained (50 ml) was measured spectrophotometrically at 600 nm by the method of Lowry et al. (42), and its esterolytic activity was evaluated [following the method of Worthington (43)] as 30 TAME Units/ml.

Preparation of Affinity Macroligand

Cross-linked STI was prepared following Bartling and Barker (21) – who modified the method originally suggested by Woodward et al. (44). 10 mg STI and 10.5 mg $NaHCO_3$ were dissolved in 2 ml ultrapure water, followed by the addition of 32.5 mg Woodward's Reagent K. The solution was kept at 25°C for 18 hr in a shaker-incubator. During this period a white precipitate was formed which was dissolved by adding 13 ml 0.1 M Tris-HCl buffer (pH 8.3) and this was further diluted up to a volume of 20 ml using the same buffer. Unreacted STI which did not crosslink with Woodward's Reagent K, was removed by ultrafiltration (at a transmembrane pressure of 294.21 kPa). The thin, tan-colored gel left as residue on the membrane surface was treated with additional 10 ml 0.1 M Tris-HCl buffer (pH 8.3) and repeated filtration was carried out until no protein appeared in the permeate thereby achieving maximum membrane activation.

Affinity Ultrafiltration

Washing was carried out at transmembrane pressures in the range 3–5 kg/cm^2 (i.e. 392.28–490.35 kPa) using pancreatic extract diluted with 0.1 M Tris-HCl buffer (pH 8.3) at different volume ratios (viz. 2:1, 1:1 and 1:4) at room temperature (ca. 30°C). Subsequently, elution was performed using 0.5 M KCl-HCl buffer (pH 4) as eluant. For both washing and elution phases, permeate fractions collected were assayed for total protein concentration and trypsin activity by the methods already stated. The washing and elution conditions used in this study are similar to that

used by Vedajnananda et al. (25), who followed one of the earliest workers in the field, viz. Bartling and Barker (21).

RESULTS AND DISCUSSIONS

The membrane hydraulic permeability, R_m, which is a measure of the permeation capacity of the membrane, may be expressed as (45),

$$J = R_m \frac{(\Delta P_{tm} - \Delta\pi)}{l} \tag{1}$$

where,
J is the volumetric permeate flux (ms^{-1}),
ΔP_{tm} is the transmembrane pressure ($kg\,cm^{-2}$),
$\Delta\pi$ is the osmotic pressure difference between the feed and the permeate phase ($kg\,cm^{-2}$), and
l is the membrane thickness (m).

Since the concentration of separable biomolecules in the system under consideration is of the order of low to very low, the osmotic pressure effect can be neglected. Consequently, Equation (1) reduces to

$$J = \frac{R_m}{l} \Delta P_{tm} \tag{2}$$

which shows that the slope of a J versus ΔP_{tm} plot (linear, as per Equation 2) will have slope R_m/l. It is a common practice to determine membrane hydraulic permeability from a plot of water flux as a function of transmembrane pressure, as shown in Fig. 1 for the Omega^{TM} polyethersulfone membrane used in this study, which yields a value of $1.8 \times 10^{-4}\,m^2s^{-1}\,(kg\,cm^{-2})^{-1}$ for R_m.

Whereas "washed off" protein mainly consists of impurities that do not bind to the affinity macroligand and consequently appear in the washing phase permeate; eluted protein, i.e. that present in the elution phase permeate, is predominantly target protein. Now, in order to study the effect of variation in feed composition on active trypsin yield and on time profiles of "washed-off" and eluted protein concentration, as well as that of the volumetric flux of washing and elution phases, ultrafiltration experiments were conducted at three different feed compositions (volumetric ratios of pancreatic extract and wash buffer in the feed maintained at 1:4, 1:1 and 2:1 respectively, corresponding to 20%, 50%, and 67% v/v of pancreatic extract) and at a transmembrane pressure of $4\,kg/cm^2$.

Comparative time profiles of volumetric flux of permeate and permeated protein concentration/throughput in the washing and the elution phases of affinity ultrafiltration are shown in Figs. 2–7. All these profiles

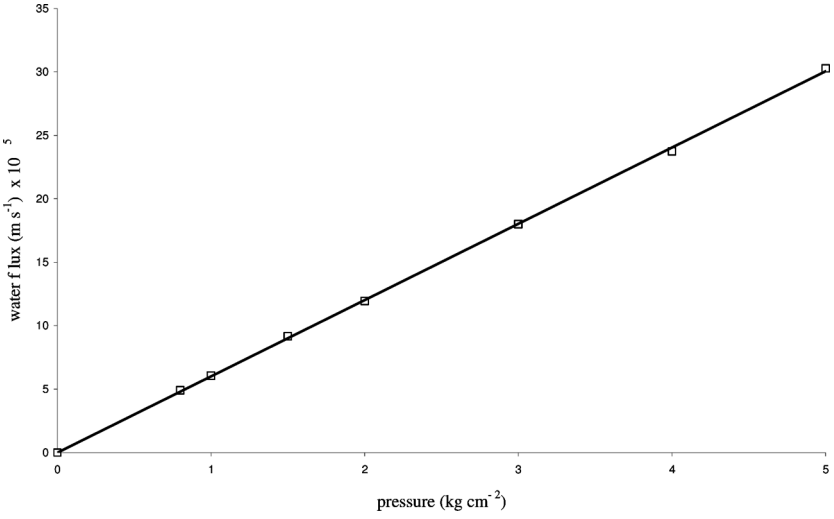

Figure 1. Water flux as a function of transmembrane pressure for Omega™ polyethersulfone membrane.

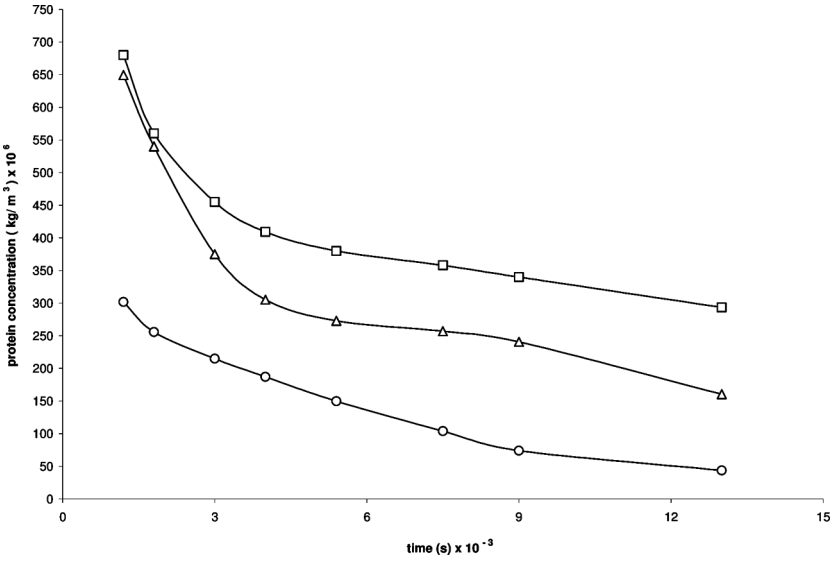

Figure 2. Comparative time profiles of washed off protein concentration for varying feed compositions at $4\,kg/cm^2$ transmembrane pressure [pancreatic extract: buffer (by volume) – 2:1 (□), 1:1 (Δ) and 1:4 (○)].

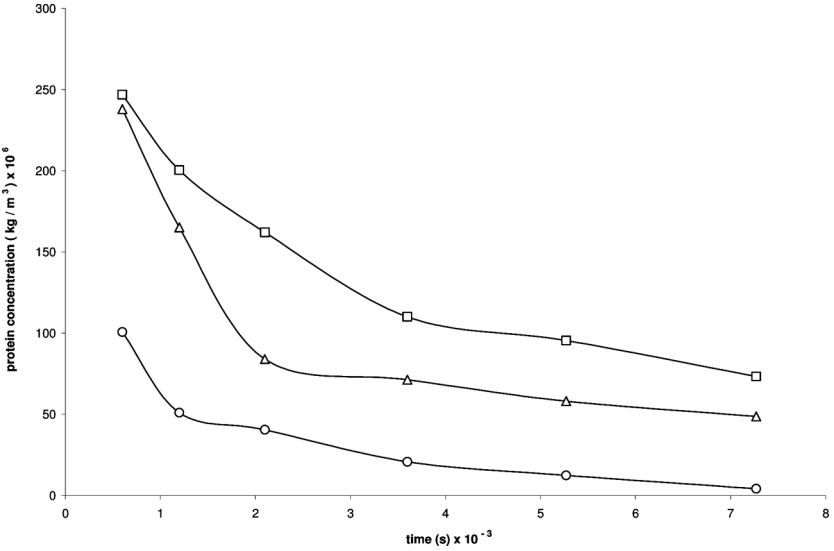

Figure 3. Comparative time profiles of eluted protein concentration for varying feed compositions at 4 kg/cm^2 transmembrane pressure [pancreatic extract: buffer (by volume) – 2:1 (□), 1:1 (Δ) and 1:4 (○)].

are characterized by a general declining trend. Now, the driving force for the membrane-mediated mass transfer of proteins from the retentate phase to the permeate phase is highest initially, and decreases progressively with permeation. Therefore the permeate flux and the protein concentration in the permeate is observed to decrease with time since flux varies directly with the driving force for mass transfer. Again, the driving force for mass transfer through the membrane decreases because the resistance to permeation increases progressively. This resistance resides not only within the membrane but also in the cake that forms on it.

From Fig. 2 it is noted that for all the three feed compositions, the time profiles of the "washed-off" protein are similar—protein concentration continuously decreases with time, the rate of decline being much sharper initially. For example, at 2:1 feed ratio the "washed-off" protein concentration drops by 40% in 40 minutes from 20 min to ca. 1 h (4000 s), but in the following 1.5 h, the decrease is only 17%. Protein concentration is consistently higher for higher concentration of pancreatic extract in feed, e.g. compared to the concentration value for 1:4 feed ratio, those for 1:1 and 2:1 feed ratios are respectively 63% and 118% higher at ca. 1 h (4000 s); and, 225% and 359% higher at 2.5 h (9000 s).

A characterizing trend observed in the time profiles of eluted protein concentration (Fig. 3) is that protein concentration in eluate falls sharply

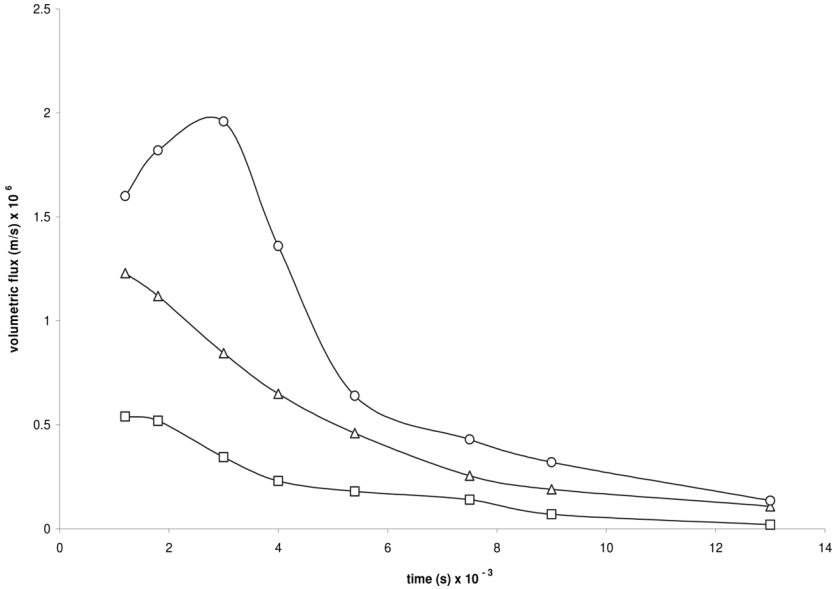

Figure 4. Comparative time profiles of volumetric flux of washing phase permeate for varying feed compositions at 4 kg/cm^2 transmembrane pressure [pancreatic extract: buffer (by volume) – 2:1 (□), 1:1 (Δ) and 1:4 (○)].

in the first hour, but gradually tends to plateau off subsequently. For example, for 1:1 feed ratio, the drop is 65% in 25 minutes from 10 min (600 s) to 35 min (2100 s), but in the next 25 minutes, i.e. up to 1 h (3600 s) the fall is only 15%. As in Fig. 2, the eluted protein concentration is also consistently higher, in fact by substantial amounts, for higher concentration of pancreatic extract in feed, e.g. relative to the value for 1:4 feed ratio, those for 1:1 and 2:1 feed ratios are respectively 110% and 300% higher at 35 min (2100 s); 250% and 450% higher at 1 h (3600 s) and, 365% and 665% higher at ca. 1.5 h (5270 s). Concentrations of ''washed-off'' protein (impurities) as well as eluted protein are both consistently higher for higher concentration of pancreatic extract in feed. This is expected, since higher levels of pancreatic extract implies higher levels of target protein (that is eluted out) as well as that of associated impurities (that are washed off) in feed, which are reflected in corresponding concentration levels of ''washed-off'' protein and eluted protein obtained during ultrafiltration.

In Fig. 4 it is observed that except for an unusual initial transient for the 1:4 feed ratio lasting for ca. 40 min during which flux increases, the time profiles of volumetric flux of washing phase permeate for all the three feed compositions show a declining trend with time, with the rate

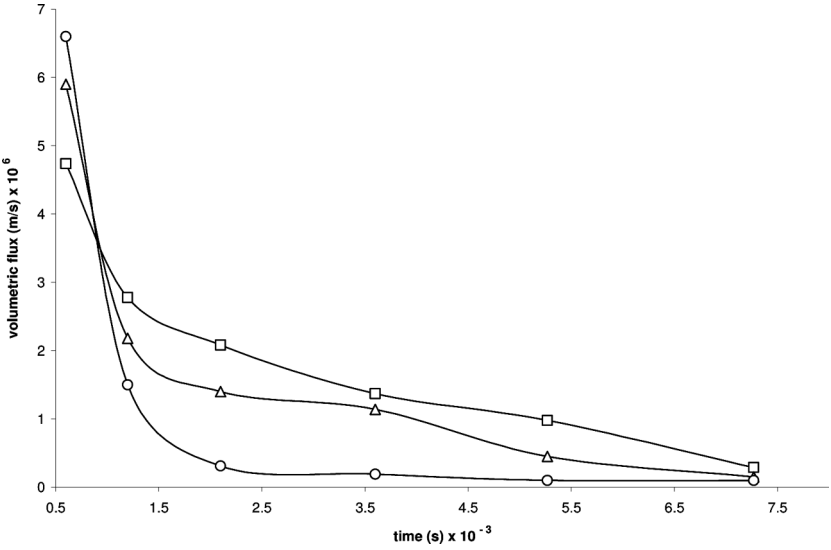

Figure 5. Comparative time profiles of volumetric flux of eluate for varying feed compositions at 4 kg/cm^2 transmembrane pressure [pancreatic extract: buffer (by volume) – 2:1 (□), 1:1 (Δ) and 1:4 (○)].

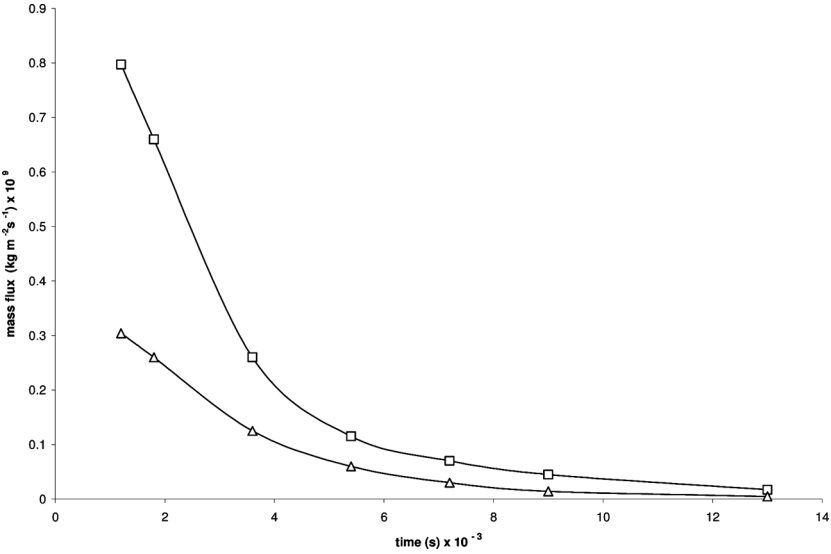

Figure 6. Effect of variation of transmembrane pressure on mass flux of "washed off" protein [4 kg/cm^2 (□), 3 kg/cm^2 (Δ); pancreatic extract: buffer – 1:1 (by volume)].

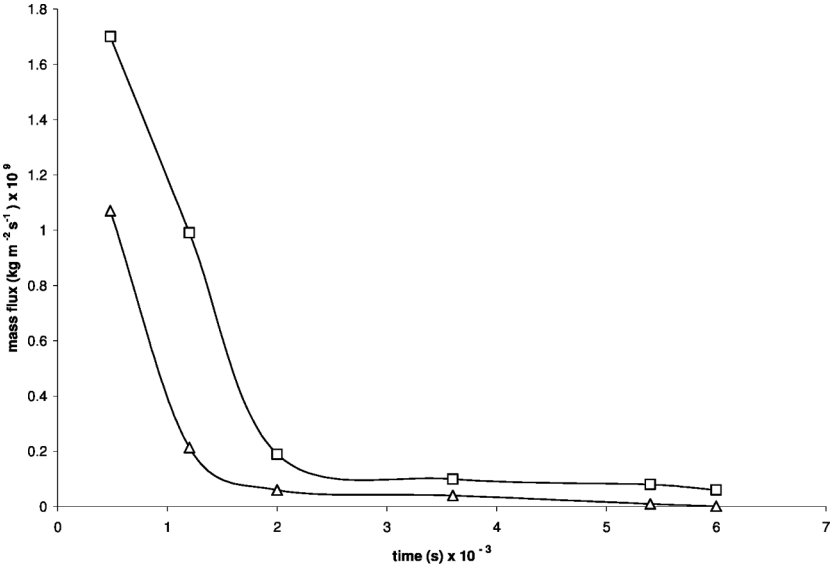

Figure 7. Effect of variation of transmembrane pressure on mass flux of eluted protein [4 kg/cm^2 (□), 3 kg/cm^2 (Δ); pancreatic extract: buffer – 1:1 (by volume)].

of decline slowly falling off. It may be noted that this initial transient occurs only for the case with the lowest protein concentration in feed (i.e. 20%) and not for the other two cases where protein concentration in feed (i.e. 50% and 67%) are much higher. With a very low solute concentration, the cake on the membrane surface takes some time to form during which the permeate flow through the membrane is practically unhindered resulting in volumetric flux increasing with time, although transiently. Once the cake has formed, it offers resistance to the permeate flow and the volumetric flux starts to decrease. For the other cases where the solute levels in feed are 2.5–3.5 times higher, the cake resistance acts almost from the start of permeate flow, therefore, no such transients are observed. At the lowest feed ratio (1:4), after the initial transient is over, there is a 67% decline in flux in the following 40 minutes, i.e. from 50 min (3000 s) to 1.5 h (5400 s), whereas during the next 1.5 h (i.e. up to 9000 s) the fall is only 50%. Unlike the profiles for "washed-off" protein, however, at corresponding time instants, flux is seen to be consistently higher for lower concentration of pancreatic extract in feed, e.g. relative to the value for the highest feed ratio (2:1), those for 1:1 and 1:4 feed ratios are respectively 145% and 468% higher at 50 min (3000 s); 155% and 255% higher at 1.5 h (5400 s) and, 171% and 357% higher at 2.5 h (9000 s). Since

resistance to permeation due to the cake formed on the membrane surface is higher for higher protein concentration in feed, therefore, at corresponding time instants, lower the feed concentration of protein, higher the volumetric flux of permeate.

The time profiles of volumetric flux of eluate (Fig. 5) on the other hand, exhibit an initial transient up to ca. 16 min (940 s) when flux is observed to be higher for lower concentration of pancreatic extract in feed (i.e. during this period flux is highest at 20% feed concentration, followed by those at 50% and 67%). Subsequently, however, this initial trend is completely reversed and flux is found to be consistently higher for higher feed concentration – e.g. relative to the value for 20% feed concentration, those for 50% and 67% feed concentrations are respectively 45% and 85% higher at 20 min; 500% and 621% higher at 1 h and, 350% and 880% higher at ca. 1.5 h (5270 s). In order to understand this phenomenon, it should be noted that at the start of elution, the cake formed on the membrane during washing is still in place. With the addition of the elution buffer and change of pH to facilitate elution, the cake gradually dissociates (breaks down). However, as long as this layer has not fully dissolved, it offers resistance to permeation which is higher for higher protein in feed (and therefore in permeate) – consequently the eluate flux is lower for higher concentration of protein in feed. Within ca. 15 min (910 s), the cake dissolves completely, and subsequently the eluate flux is found to be higher for higher protein concentration in feed.

Keeping the transmembrane pressure constant and examining the effects of variation in feed composition on volumetric flux and protein concentration profiles of the respective permeates of washing and elution phases it appears that there is no "optimum" feed composition at which all the studied performance variables are concurrently maximized. Now, determination of trypsin activity in permeates of both washing and elution phases helps to quantify, respectively,

1) the amount of trypsin unbound to the affinity ligand that escapes with the "washed off" non-target proteins/impurities, and
2) the "active trypsin" yield which expresses the target enzyme recoverable in eluate as a fraction of active trypsin originally present in the feed to washing phase.

Whereas the former is an indicator of the efficiency of binding of the target enzyme to the affinity ligand (and has a direct bearing on the overall separation efficiency) the latter i.e. active trypsin yield – determined by comparative evaluation of trypsin activity in eluate and feed, is perhaps the key quantitative indicator of the overall separation efficiency.

In the present study, it was found that no active trypsin was detectably present in washing phase permeate for all the process conditions investigated. Clearly, this points to the excellent binding efficiency of the target enzyme i.e. trypsin with the chosen affinity ligand i.e. STI. In fact, insofar as trypsin sourced from goat pancreas is concerned, this result confirms the appropriateness of selection of the combination of wash buffer and affinity ligand used in this study.

With regard to the active trypsin yield, it was found that for all combinations of feed composition and TMP considered, the yield is around 70% with a maximum of 73.9% obtained for feed with 50% pancreatic supernatant at 4 kg/cm^2 transmembrane pressure. This, notably, is comparable to the 77% trypsin yield reported earlier by Luong et al. (33) with porcine pancreas. The values of active trypsin yield corresponding to 20%, 50%, and 67% v/v of pancreatic supernatant in feed at 4 kg/cm^2 transmembrane pressure are 69.4%, 73.9%, and 65.9% respectively – with a clear maximum for 50% feed composition.

Once the optimum feed composition is identified (with regard to resolution, i.e. active trypsin yield), the effect of variation in transmembrane pressure, at this optimum feed composition, on process performance, is to be investigated in accordance with our stated objectives. It may be noted here that although it was intended to vary the transmembrane pressure both above and below 4 kg/cm^2, preliminary runs at 5 kg/cm^2 showed that experimentation at this transmembrane pressure would not be productive and instead could cause membrane damage. Accordingly, comparative evaluation of process performance (based on mass flux profiles of washed-off and eluted proteins – noting that mass flux being the product of volumetric flux of permeate and protein concentration therein serves the purpose of evaluating process performance in terms of a single process variable) and active trypsin yield at 3 kg/cm^2 and 4 kg/cm^2 transmembrane pressure at 1:1 feed composition was carried out. Changing the transmembrane pressure from 4 kg/cm^2 to 3 kg/cm^2 at this feed composition results in a decrease in yield from 73.9% to 69.6%.

From Fig. 6 depicting the comparative time profiles of the ''washed-off'' protein mass flux at two different transmembrane pressures i.e. 3 kg/cm^2 and 4 kg/cm^2 (at 1:1 feed ratio) it is found that mass flux at 4 kg/cm^2 always remains higher than that for 3 kg/cm^2 e.g. 162% higher at 20 min, 108% at 1 h and 92% at 1.5 h. With regard to the rate of flux decline, it is observed that at both the transmembrane pressures considered, the mass flux decreases drastically in the initial period (in a 40 min interval from 20 min to 1 h the drop in mass flux is 67% and 59% respectively at 4 kg/cm^{2}and 3 kg/cm^2 transmembrane pressures) and then declines somewhat steadily.

The time profiles of eluted protein mass flux at two different transmembrane pressures (at 1:1 feed ratio) are shown in Fig. 7, and are qualitatively similar to those in Fig. 6-eluted protein mass flux is higher at higher transmembrane pressure e.g. 363% higher at 20 min, 216% at ca. 0.5 h (2000 s) and 150% at 1 h; again, as far as rate of flux decline is concerned, there is an initial drastic fall in the first 30 minutes, and subsequent plateauing off. Thus, comparative mass flux profiles at the two transmembrane pressure values for both the "washed-off" and eluted proteins exhibit a consistent trend – higher mass flux for higher transmembrane pressure at corresponding time instants. Although flux profiles are presented here for 50% feed composition, similar trends were observed at the other feed compositions investigated. These trends are not unexpected, since a higher transmembrane pressure provides a higher driving force for mass transfer causing greater permeation of protein ("washed off"/eluted protein, as the case may be).

CONCLUSION

The Comparative concentration profiles of "washed-off" and eluted protein at the three feed compositions studied show that concentrations of "washed-off" protein (impurities) as well as eluted protein are both consistently higher for higher concentration of pancreatic extract in feed. However, as far as comparative volumetric flux profiles are concerned, while those of the eluate are consistently higher for higher concentration of pancreatic extract in feed, in contrast, the exact opposite occurs in the washing phase i.e. higher the concentration of pancreatic extract in feed, lower the flux. No trypsin activity is found to be detectably present in the washing phase permeate for all process conditions considered, thus, confirming excellent binding of the target enzyme i.e. trypsin with the chosen affinity ligand i.e. cross-linked STI. Again, the values of the active trypsin yield corresponding to feed ratios (v/v) 1:4, 1:1, and 2:1 of pancreatic extract in feed at 4 kg/cm^2 transmembrane pressure are 69.4%, 73.9% and 65.9%, respectively – with a clear maximum for 1:1 feed composition. Evidently, at the optimum feed ratio (i.e. 1:1), the ultrafiltration process performance evaluated in terms of active trypsin yield on the one hand, and mass flux profiles of eluted protein (i.e. target product) and washed-off protein (i.e. impurities) on the other; are all found to be favored at the higher transmembrane pressure, i.e. 4 kg/cm^2. From these results it may be concluded that the separation performance of the affinity ultrafiltration of goat pancreatic trypsin, using the combination of affinity ligand, wash buffer, elution buffer, and other micro-environmental conditions employed in this study, may be optimized at 1:1 feed ratio and 4 kg/cm^2 transmembrane pressure.

REFERENCES

1. Brown, W.E.; Wold, F. (1973) Alkyl isocyanates as active-site-specific reagents for serine proteases. Identification of the active-site serine as the site of reaction. *Biochemistry*, *12* (5): 835–840.
2. ProdeGene Seedquest News Releases, College Station, Texas, February 13, 2002.
3. Kunitz, M. (1947) Crystalline soybean trypsin inhibitor II. General properties. *J. Gen. Physiol.*, *30*: 291–310.
4. Travis, J.; Liener, I.E. (1965) The crystallization and partial characterization of porcine trypsin. *J. Biol. Chem.*, *240*: 1962–1966.
5. Buck, F.F.; Vithayathil, A.J.; Bier, M.; Nord, F.F. (1962) On the mechanism of enzyme action. LXXIII. Studies on trypsins from beef, sheep and pig pancreas. *Arch. Biochem. Biophys.*, *97*: 417–424.
6. Travis, J. (1968) Studies on the active sites of sheep trypsin. *Biochem. Bioph. Res. Co.*, *30*: 730–734.
7. Das, S.K.; Sinha, N.K. (1972) Goat trypsin: Purification, properties & amino acid composition. *Indian J. Biochem. Bio.*, *9*: 287–292.
8. Stevenson, K.; Voordouw, J. (1975) Characterization of trypsin and elastase from the moose (*Alces alces*). I. Amino acid composition and specificity towards polypeptides. *Biochim. Biophys. Acta*, *386* (1): 324–331.
9. Bricteux-Gregoire, S.; Schyns, R.; Florkin, M.; Emmens, R.; Welling, G.; Beintema, J. (1975) N-Terminal amino acid sequence of trypsinogen from the lesser rorqual *Balaenoptera acutorostrata* (*Cetacea*). Simultaneous isolation of trypsinogen, chymotrypsinogen and ribonuclease from pancreas. *Biochim. Biophys. Acta*, *386* (1): 244–255.
10. Bricteux-Gregoire, S.; Schyns, R.; Florkin, M. (1974) N-Terminal amino acid sequence of trypsinogen from the elephant seal *Mirounga leonina* L. (Carnivora). *Biochim. Biophys. Acta*, *351*: 87–91.
11. Reeck, G.; Neurath, H. (1972) Pancreatic Trypsinogen from the African Lungfish. *Biochemistry*, *11* (4): 503–510.
12. Hermodson, M.; Ericsson, L.; Neurath, H.; Walsh, K. (1973) Determination of the amino acid sequence of porcine trypsin by sequenator analysis. *Biochemistry*, *12* (17): 3146–3153.
13. Walker, J.; Keil, B. (1973) Purification and characterization of different active forms of pork trypsin. *Eur. J. Biochem.*, *32*: 486–491.
14. Vandermeers, A.; Vandermeers-Piret, M.; Rathe, J.; Christophe, J. (1973) Purification, potential enzymatic activity and amino acid composition of chymotrypsinogen and trypsinogens from rat pancreas. *Arch Biochem. Biophys.*, *159*: 492.
15. Ryan, C.A.; Clary, J.J.; Tomimatsu, Y. (1965) Chicken chymotrypsin and turkey trypsin. Part II: Physical and enzymological properties. *Arch Biochem. Biophys.*, *110*: 175–183.
16. Kishida, Trypsin; Liener, I.E. (1968) Further characterization of turkey trypsin: End groups and sequence of histidine-containing peptides. *Arch. Biochem. Biophys.*, *126* (1): 111–120.

17. Mallory, P.A.; Travis, J. (1973) Human pancreatic enzymes. Characterization of anionic human trypsin. *Biochemistry*, *12* (15): 2847–2851.
18. Figarell, C.; Negri, G.; Guy, O. (1975) The two human trypsinogens. Inhibition spectra of the two human trypsins derived from their purified zymogens. *Eur. J. Biochem.*, *53*: 457–463.
19. Travis, J.; Roberts, R.C. (1969) Human trypsin. Isolation and physical-chemical characterization. *Biochemistry*, *8*: 2884–2889.
20. Sinha, N.K.; Das, S.K. (1970) Crystallization & Some properties of goat trypsin. *Indian J. Biochem.*, *7*: 78–80.
21. Bartling, G.J.; Barker, C.W. (1976) Separation of trypsin and peroxidase by ultrafiltration using crosslinked soybean trypsin inhibitor. *Biotechnol. Bioeng.*, *18* (7): 1023–1027.
22. Adamski-Medda, D.; Nguyen, Q.T.; Dellacherie, E. (1981) Biospecific ultrafiltration: A promising purification technique for proteins? *J. Membrane Sci.*, *9* (3): 337–342.
23. Choe, T.B.; Masse, P.; Verdier, A. (1986) Separation of trypsin from trypsin α-chymotrypsin mixture by affinity ultrafiltration. *Biotechnol. Lett.*, *8* (3): 163–168.
24. Luong, J.H.T.; Male, K.B.; Nguyen, A.L. (1988) Synthesis and characterization of a water soluble affinity polymer for trypsin purification. *Biotechnol. Bioeng.*, *31* (5): 439–446.
25. Vedajnananda, S.; Chowdhury, R.; Bhattacharya, P. (2001) Mathematical modelling of the washing phase of a semi-batch affinity ultrafiltration system for enzyme separation. *Biochem. Eng. J.*, *9* (1): 41–48.
26. Male, K.B.; Luong, J.H.T.; Nguyen, A.L. (1990) Isolation of urokinase by affinity ultrafiltration. *Biotechnol. Bioeng.*, *35* (1): 87–93.
27. Glatz, Z.; Novak, P. (1996) Application of affinity ultrafiltration for purification of egg-white lysozyme. *Chem. Listy.*, *90* (7): 728–729.
28. Romero, J.R.; Zydney, A. L. (2001) Chiral separations using ultrafiltration with a stereoselective binding agent. *Sep. Sci. Technol.*, *36* (7): 1575–1594.
29. Romero, J.R.; Zydney, A.L. (2002) pH and salt effects on chiral separations using affinity ultrafiltration. *Desalination*, *148* (1): 159–164.
30. Romero, J.R.; Zydney, A.L. (2002) Staging of affinity ultrafiltration processes for chiral separations. *J. Membrane Sci.*, *209* (1): 107–119.
31. Romero, J.R.; Zydney, A.L. (2002) Affinity ultrafiltration: Effect of ligand binding on selectivity and process optimization. *Biotechnol. Bioeng.*, *77* (3): 256–265.
32. Rao, S.; Zydney, A.L. (2002) High resolution protein separations using affinity ultrafiltration with small charged ligands. *J. Membrane Sci.*, *280* (1–2): 781–789.
33. Luong, J.H.T.; Male, K.B.; Nguyen, A.L. (1988) A continuous affinity ultrafiltration process for trypsin purification. *Biotechnol. Bioeng.*, *31* (6): 516–520.
34. Lebrun, F.; Bazus, A.; Dhulster, P.; Guillochon, D. (1988) Influence of molecular interactions on ultrafiltration of a bovine hemoglobin hydrolysate with an organic membrane. *J. Membrane Sci.*, *146* (1): 113–124.

35. Cowan, S.M.; Ritchie, S.M. (2006) Enhancement of Whey Protein Filtration Using Polyethersulfone Membranes. AIChE Annual Meeting on Membrane-Based Separations – Poster Session on Membranes, San Francisco, USA.
36. Cheang, B.; Zydney, A.L. (2003) Separation of alpha-lactalbumin and beta-lactoglobulin using membrane ultrafiltration. *Biotechnol. Bioeng.*, *83* (2): 201–209.
37. Makdissy, G.; Croué, J.P.; Amy, G.; Buisson, H. (2004) Fouling of a polyethersulfone ultrafiltration membrane by natural organic matter. *Water Sci. Technol: Water Supply*, *4* (4): 205–212.
38. Priyananda, P.; Chen, V. (2003) *Ultrafiltration of Protein and Fatty Acid Mixtures*. 5th IMSTEC (International Membrane Science and Technology Conference): Sydney, Australia.
39. Boyd, R.F.; Zydney, A.L. (1998) Analysis of protein fouling during ultrafiltration using a two-layer membrane model. *Biotechnol. Bioeng.*, *59* (4): 451–460.
40. Rahimpour, A.; Madaeni, S.S.; Mansourpanah, Y. (2007) High performance polyethersulfone ultrafiltration membrane for manufacturing spiral wound module: Preparation, morphology, performance and chemical cleaning. *Polym. Advan. Technol.*, *18* (5): 403–410.
41. Lecco, F.L.; Vicenza, C.R.; Mirandola, C.T. (ed.) (2003) *Polyethersulfone: Membranes for Multiple Clinical Applications, Contributions to Nephrology*, Vicenza, C.R., ed.; S. Karger AG: Basel, Switzerland, 138.
42. Lowry, O.H.; Rosebrough, N.J.; Farr, L.; Randall, R.J. (1951) Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.*, *193*: 265–275.
43. Worthington, V. (1993) In: *Worthington Enzyme Manual*, Worthington, V., ed.; Worthington Biochemical: Freehold, NJ, 374–381.
44. Woodward, R.B.; Olofson, R.A.; Mayer, H. (1961) A New Synthesis of Peptides. *J. Am. Chem. Soc.*, *83*, 1010–1012.
45. Viker, S. (1961) In: *Membrane Handbook*, Ho, W.S.W.; Sirkar, K.K., eds.; Van Nostrand Reinhold: NY.