

**STUDY OF LIMITING FLUX FOR MIXTURE OF PROTEINS  
DURING ULTRAFILTRATION**

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**STUDY OF LIMITING FLUX FOR MIXTURE OF PROTEINS  
DURING ULTRAFILTRATION**

by

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## **CERTIFICATE**

This is to certify that the thesis entitled “**Study of limiting flux for mixture of proteins during ultrafiltration**” being submitted by Mr. **Sunil Kumar** is worthy of consideration for the award of the degree of **Doctor of Philosophy**. The thesis has been prepared by him under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi and is a record of the original bonafide research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

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Einstein quotes that 'learning is an experience, everything else is just information', I hope this experience of passing through the hardships and self-realisation of one's potential helps me in giving back 'something worthwhile' to the society.

**Sunil Kumar**

## ABSTRACT

Ultrafiltration is being used increasingly for concentration and purification of macromolecules like proteins and monoclonal antibodies. High flux membranes with accurate selectivity are desirable in such applications. At higher pressure, the flux becomes independent of the applied pressure and this state is called limiting flux. The general notion is that no permeation of solute occurs after the onset of limiting flux, and is considered as a bottleneck for the process. During ultrafiltration of mixture of macro-solutes, in which one was partially retained, and another one was completely retained, transmission in the limiting flux regime was observed. This research opened a new window to study the phenomena of limiting flux with a different perspective.

In the present work, ultrafiltration experiments were performed on two different modules; dead-end stirred cell modules (low-pressure range till 1 bar) and cross-flow module (for higher pressure ranges up to 10 bar). Commercially available high flux polymeric membranes, of various molecular weight cut-offs (MWCO) ranging between 5 kDa to 100 kDa were used during the ultrafiltration of proteins namely  $\gamma$ -Globulin, Lysozyme, Bovine Serum Albumin (BSA), Myoglobin and binary mixtures of  $\gamma$ -Globulin with Lysozyme and BSA with Myoglobin. Polyethylene Glycols (PEG35, PEG3) and their binary mixtures were also used. The flux attained limiting values at very low transmembrane pressures in stirred cell module while in crossflow, the pressure-independent regime was attained at higher pressures. Also, the lower molecular weight macro-solutes, proteins or PEG, did not attain limiting flux in the pressure range ultrafiltration was carried out. Complete retention of  $\gamma$ -Globulin, BSA, PEG35 was found while lysozyme, myoglobin, PEG3 still permeated out even after the onset of limiting flux during the ultrafiltration of binary mixtures. Permeation of low molecular weight macro-solutes in the pressure-independent regime has been observed and reported for the first time. Various concentrations of  $\gamma$ -Globulin+Lysozyme in their mixture were used to explore the extent of protein transmission after the attainment of limiting flux. Limiting flux was predominantly controlled by the concentration of  $\gamma$ -Globulin. However, comparison of transmission obtained for pure lysozyme and its mixture with  $\gamma$ -Globulin showed dramatic results where lysozyme transmission in the mixture at the onset of limiting flux was observed to be higher than that of the one when pure lysozyme was filtered.  $\gamma$ -Globulin also facilitated the lysozyme transmission in the mixture till it attained limiting flux. BSA of concentration range up to 100 g/l was also used for evaluating the behaviour of limiting regime in higher concentration region. BSA+Myoglobin binary mixture was also

used to study the effect on transmission because of attainment of limiting flux. Since the solubility limits of PEGs are high as compared to proteins, use of PEGs was considered on the basis that their high concentrations solutions (near to the calculated gel concentrations) could be prepared with ease. PEG35 and PEG3 (concentration up to 300 g/l) and their binary mixture were also used in cross-flow ultrafiltration. Unlike proteins, PEGs are neutral molecules, and because of this, transmission of PEG 3 in the mixture was found to be less as compared to the protein transmission.

This work provides insights into the occurrence of limiting flux during ultrafiltration of proteins. The onset of limiting flux has been attributed to the increased concentration of the rejected proteins at the membrane surface resulting in the formation of so-called ‘gel-like layer’ onto the membrane. Here, the gel has been considered as a matrix whose concentration varied along the thickness. The change in concentration enabled the existence of pores, large enough for the permeation of smaller solutes (proteins or PEG) in the binary mixture of higher molecular weight solutes (proteins or solutes). Since higher molecular weight proteins (HMWP) are completely retained, they get polarized at the membrane surface while lower molecular weight proteins (LMWP) were partially retained, and this layer of the large HMWP-LMWP-buffer-water matrix has been envisaged as supra-structure of multilayer membrane-like structure contributing to increasing osmotic pressure. Transmission of the low molecular weight macro-solutes even in the limiting regime, controlled by the higher molecular weight solute, has been the most revealing part. The findings are contrary to the general perception that onset of limiting flux changes the permeation characteristics of the membrane considerably. The methodology used here could be adapted to analyze the formation of concentration polarized layer more intensely by analysing the surface characteristics of this so-called gel-like matrix. This information could also be vital for protein separation even in the pressure-independent regime which was generally not considered as a possible alternative.

## सार

प्रोटीन और मोनोक्लोनल एंटीबॉडी जैसे मैक्रोमोल्यूलस की सांद्रता और शुद्धिकरण के लिए अल्ट्राफिल्ट्रेशन का बहुतायत में उपयोग किया जा रहा है। परिशुद्ध चयन करने वाले उच्च प्रवाह झिल्ली ऐसे अनुप्रयोगों में वांछनीय हैं। उच्च दबाव पर झिल्ली से निकलने वाला प्रवाह अनुप्रयुक्त दबाव से स्वतंत्र हो जाता है। प्रवाह की इस दशा को सीमित-प्रवाह कहा जाता है। सामान्य धारणा यह है कि प्रवाह के सीमित होते ही कोई पारगम्यता नहीं होती है और तब प्रक्रिया के दौरान प्रवाह का सीमित होना एक बाधा के रूप में माना जाता है। प्रस्तुत कार्य में मैक्रो-सोल्यूट्स के मिश्रण का चयन इस प्रकार किया गया कि एक आंशिक रूप से बचा रहे और दूसरा पूरी तरह से झिल्ली द्वारा खारिज हो जाये। मिश्रण के अल्ट्राफिल्ट्रेशन के दौरान सीमित-प्रवाह स्थिति में संचरण की खोज पहली बार की गयी है। इससे इस शोध ने एक अलग परिप्रेक्ष्य के साथ प्रवाह के सीमित होने की घटना का अध्ययन करने के लिए एक नई राह दिखायी है।

प्रस्तुत कार्य में, दो अलग-अलग मॉड्यूल पर अल्ट्राफिल्ट्रेशन प्रयोग किए गए हैं; गतिरोध उत्तेजित सेल मॉड्यूल (निम्न दबाव के लिए: १ बार दबाव तक) और क्रॉस-फ्लो मॉड्यूल (उच्च दबाव के लिए: १० बार दबाव तक)। ५ kDa से १०० kDa के बीच विभिन्न मॉलिक्यूलर-वेट-कट-ऑफ (एम.डब्ल्यू.सी.ओ) के उच्च प्रवाह वाली बहुलक झिल्ली, जो वाणिज्यिक रूप से उपलब्ध हैं, उनका इस्तेमाल प्रोटीन के अल्ट्राफिल्ट्रेशन के दौरान किया गया है जिनमें  $\gamma$ -ग्लोब्युलिन, लाइसोजाइम, बोवाइन सीरम एल्ब्यूमिन (बी.एस.ए), मायोग्लोबिन और इनके बाइनरी मिश्रण (अर्थात्  $\gamma$ -ग्लोब्युलिन के साथ लाइसोजाइम और बी.एस.ए के साथ मायोग्लोबिन) सम्मिलित हैं। पोलिथीलीन ग्लाइकोल्स (PEG ३५, PEG ३) और उनके बाइनरी मिश्रण का भी उपयोग किया गया है। गतिरोध उत्तेजित सेल मॉड्यूल में बहुत कम ट्रांसमम्ब्रेन दबावों पर प्रवाह ने अपने सीमित अंकों को प्राप्त किया जबकि क्रॉसफ्लो मॉड्यूल में प्रवाह ने अपने सीमित अंकों को उच्च दबाव पर प्राप्त किया। इसके अलावा, निम्न आणविक भार मैक्रो-सोल्यूट्स, प्रोटीन या PEG ने अल्ट्राफिल्ट्रेशन के दौरान लगाए गए दबाव सीमा में सीमित-प्रवाह को प्राप्त नहीं किया।  $\gamma$ -ग्लोब्युलिन, बी.एस.ए एवं PEG ३५ का सम्पूर्ण प्रतिधारण पाया गया था, जबकि लाइसोजाइम, मायोग्लोबिन, PEG ३ बाइनरी मिश्रणों के अल्ट्राफिल्ट्रेशन के दौरान, प्रवाह सीमित होने के बाद भी बाहर निकल रहे थे। दबाव-मुक्त स्थिति में निम्न आणविक भार मैक्रो-सोल्यूशन की पारगम्यता पहली बार देखी गई है और इस पर प्रकाश डाला गया है। अपने मिश्रण में  $\gamma$ -ग्लोब्युलिन एवं लाइसोजाइम की विभिन्न सांद्रता का उपयोग प्रवाह को सीमित करने की प्राप्ति के बाद प्रोटीन संचरण की सीमा का पता लगाने के लिए किया गया है। सीमित-प्रवाह को मुख्य रूप से  $\gamma$ -ग्लोब्युलिन की सांद्रता द्वारा नियंत्रित किया गया है। हालांकि, जब शुद्ध लाइसोजाइम फ़िल्टर किया गया था शुद्ध लाइसोजाइम के लिए प्राप्त संचरण की तुलना और  $\gamma$ -ग्लोब्युलिन के साथ इसके मिश्रण ने अप्रत्याशित निष्कर्ष निकले जहां प्रवाह के सीमित होते समय ही मिश्रण में लाइसोजाइम ट्रांसमिशन को उस समय की तुलना में अधिक पाया गया।  $\gamma$ -ग्लोब्युलिन ने मिश्रण में लाइसोजाइम संचरण को सुगम बनाया जब तक यह मिश्रण अपने अल्ट्राफिल्ट्रेशन के दौरान सीमित-प्रवाह को प्राप्त नहीं कर लेता। उच्च सांद्रता क्षेत्र में सीमित प्रवाह के व्यवहार का मूल्यांकन करने के लिए १०० ग्राम प्रति लीटर तक सांद्रता का बी.एस.ए का भी इस्तेमाल किया गया है। बी.एस.ए एवं मायोग्लोबिन बाइनरी मिश्रण का भी उपयोग किया गया है एवं मायोग्लोबिन ट्रांसमिशन पर सीमित-प्रवाह के प्रभाव का अध्ययन भी किया गया है। चूंकि प्रोटीन की तुलना में PEG की घुलनशीलता सीमा अधिक होती है, इसलिए PEG का भी उपयोग उच्च सांद्रता तक किया गया है।



इसका आधार ये माना गया था कि इनके उच्च सांद्रता मिश्रण को जैल सांद्रता के नजदीक तक आसानी से तैयार किए जा सकता है। PEG ३५ और PEG ३ के ३०० ग्राम प्रति लीटर तक की सांद्रता और उनके बाइनरी मिश्रण का प्रयोग क्रॉस-फ्लो अल्ट्राफिल्ट्रेशन में भी किया गया है। प्रोटीन के विपरीत, PEG तटस्थ अणु होते हैं, और इसके कारण, मिश्रण में PEG ३ का संचरण प्रोटीन संचरण की तुलना में कम पाया गया था।

यह कार्य प्रोटीन के अल्ट्राफिल्ट्रेशन के दौरान सीमित-प्रवाह की घटना में अंतर्दृष्टि प्रदान करता है। प्रवाह के सीमित होने के शुरुआत से ही झिल्ली सतह पर खारिज प्रोटीन की सांद्रता बढ़ती जाती है जिसके परिणामस्वरूप झिल्ली पर तथाकथित 'जैल जैसी परत' का गठन होता है। यहां, जैल को एक मैट्रिक्स के रूप में माना गया है जिसका घनत्व सांद्रता के साथ भिन्न होता जाता है। सांद्रता में परिवर्तन के कारण छिद्रों का अस्तित्व संभव हुआ, जो उच्च एवं निम्न आणविक भार प्रोटीन की बाइनरी मिश्रण में छोटे विलेय (प्रोटीन या PEG) के पारगम्य के लिए काफी बड़ा पाया गया। चूंकि उच्च आणविक भार प्रोटीन (एच.एम.डब्ल्यू.पी) झिल्ली सतह पर पूरी तरह से खारिज हुए, इसलिए वे झिल्ली की सतह पर ध्रुवीकरण प्राप्त करते गए जबकि निम्न आणविक वजन प्रोटीन (एल.एम.डब्ल्यू.पी) आंशिक रूप से झिल्ली सतह पर खारिज हुए, इससे एक बड़े एच.एम.डब्ल्यू.पी-एल.एम.डब्ल्यू.पी-बफर-पानी की मैट्रिक्स की एक परत को अतिउच्च संरचना के रूप में देखा गया है। बहुआयामी झिल्ली-जैसी संरचना के निर्माण से यहां परासरणी दबाव की वृद्धि हुई। उच्च आणविक भार विलाप द्वारा नियंत्रित सीमित व्यवस्था में कम आणविक वजन मैक्रो-सोल्यूशंस का संचरण एक बहुमूल्य प्रत्यक्ष है। इस शोध का निष्कर्ष सामान्य धारणा के विपरीत है। प्रवाह के सीमित होने के बाद झिल्ली की पारगम्य विशेषतायें काफी बदल जाती हैं। इस तथाकथित जैल जैसी मैट्रिक्स की सतह की विशेषताओं का विश्लेषण करके यहां उपयोग की जाने वाली पद्धति को सांद्र-ध्रुवीकरण परत के गठन का अधिक तीव्रता से विश्लेषण करने के लिए अपनाया जा सकता है। यह जानकारी प्रोटीन पृथक्करण के लिए दबाव-मुक्त स्थिति में भी महत्वपूर्ण है जिसे आम तौर पर संभावित विकल्प के रूप में नहीं माना जाता था।

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## LIST OF ABBREVIATIONS

MWCO	Molecular weight cut-off
TMP	Transmembrane pressure
UF	Ultrafiltration
BSA	Bovine serum albumin
HPLC	High-performance liquid chromatography
PES	Polyethersulphone
PAN	Polyacrylonitrile
ATR-FTIR	Attenuated total reflectance – Fourier transform infrared spectroscopy
AFM	Atomic-force Microscope
SEM	Scanning Electron Microscope