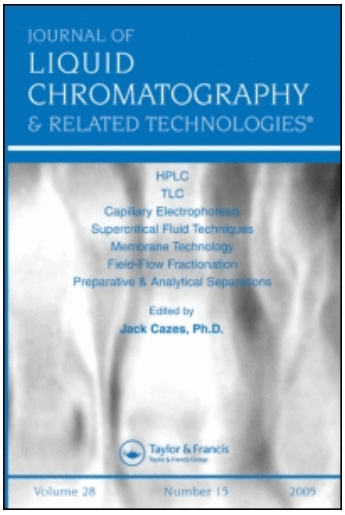

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SHEAR STRESS AND PRECIPITATION EFFECT ON ENZYMATIC ACTIVITY OF α-CHYMOTRYPSIN IN CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY

Terasut Sookkumnerd[a]; Peggy P. Hsu[a]; James T. Hsu[a]; Yoichiro Ito[b]
[a] Lehigh University, Bethlehem, PA, U.S.A. [b] Laboratory of Biophysical Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Online publication date: 30 April 2001

To cite this Article Sookkumnerd, Terasut , Hsu, Peggy P. , Hsu, James T. and Ito, Yoichiro(2001) 'SHEAR STRESS AND PRECIPITATION EFFECT ON ENZYMATIC ACTIVITY OF α-CHYMOTRYPSIN IN CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 24: 7, 947 — 955
To link to this Article: DOI: 10.1081/JLC-100103421
URL: http://dx.doi.org/10.1081/JLC-100103421

J. LIQ. CHROM. & REL. TECHNOL., 24(7), 947–955 (2001)

SHEAR STRESS AND PRECIPITATION EFFECT ON ENZYMATIC ACTIVITY OF α-CHYMOTRYPSIN IN CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY

Terasut Sookkumnerd,[1] Peggy P. Hsu,[1] James T. Hsu,[1,*] and Yoichiro Ito[2]

[1] Biopharmaceutical Technology Institute, Department of Chemical Engineering, Lehigh University, Bethlehem, PA 18015, USA
[2] Laboratory of Biophysical Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

ABSTRACT

The result of a systematic investigation into the effect of shear stress and precipitation in the separation channel of centrifugal precipitation chromatography on the enzymatic activity of α-chymotrypsin at room temperature is reported. From the experiment, it was observed that more than 90% of α-chymotrypsin remains active after being subjected only to the shear stress in the separation channel, with the exception of α-chymotrypsin subjected to a relatively high water flow rate.

Upon introducing the ammonium sulfate gradient in the separation channel, it was observed that 89.03% of the enzyme

*Corresponding author. E-mail: jth0@lehigh.edu

remained active after being subjected to both shear stress and precipitation in centrifugal precipitation chromatography. As a result, it can be concluded that centrifugal precipitation chromatography should be friendly to most proteins under the appropriate operating conditions.

INTRODUCTION

Although several new techniques in protein purification have evolved over the past three decades, ammonium sulfate precipitation is still the preferred method for crude protein purification, especially in large-scale separation.[1,2] Ammonium sulfate precipitation is, perhaps, the most inexpensive and simplest technique available and yet it does not harm most proteins and enzymes.[3] These advantages, combined with a relatively high degree of recovery, make the technique extremely useful. Unfortunately, because the solubility of proteins in ammonium sulfate solution often differs only slightly, a common batch-process, ammonium sulfate precipitation, usually does not yield high-purity proteins.

In order to improve the purity of fractionated proteins, a method of ammonium sulfate precipitation in a chromatographic column, sometimes called "zone precipitation," was developed.[4,5] Unfortunately, the efficiency of zone precipitation was reduced because of adsorption of proteins on the solid support and premature elution of smaller protein precipitates.

Recently, Ito[6] has proposed an alternative technique, called "centrifugal precipitation chromatography," to perform ammonium sulfate precipitation in a chromatographic mode. Principles of the technique, equipment, and preliminary studies on operating parameters have been discussed in detail by Ito[6,7] and Sookkumnerd et al.[8] Briefly, in this technique, a transfer of ammonium sulfate across a parallel-flow membrane unit is used to form an ammonium sulfate gradient inside the separation channel under the centrifugal force field. When injected into this separation channel, the proteins precipitate along the length of the channel, in accordance with their solubility in ammonium sulfate solution. After the proteins precipitate, an ammonium sulfate gradient in the separation channel is shifted toward the outlet. Simultaneously, the protein precipitates move with the gradient until they elute out from the separation channel. Using this strategy, mixtures of proteins,[6,8] recombinant protein,[7] and PEG-protein conjugates[9] have been successfully purified.

Although a preliminary experimental study into the effect of operating parameters on the efficiency of the protein fractionation,[7] as well as a theoretical analysis of ammonium sulfate gradient formation in the separation channel[8] have been reported, there has not been a systematic investigation of the effect of cen-

trifugal precipitation chromatography, particularly shear stress and precipitation, on enzymatic activity of proteins. Before centrifugal precipitation chromatography can be fully utilized in large-scale protein purification, such a study must be performed. While it is well known that ammonium sulfate solution is friendly to proteins and other biomolecules,[3,10] shear stress has been documented to cause losses in protein activity.[11,12]

Since it has been reported that the shear stress caused by fluid flow can sometimes denature proteins, the effect of rigorous mixing in the separation channel, caused by the combination of fluid flow and centrifugation, on the enzymatic activity of α-chymotrypsin is evaluated in this research article. In the experiment, α-chymotrypsin is chosen as a protein model due to its availability and its well-documented enzymatic assays. By varying the flow rate of water in the separation channel and the rotational speed of the separation apparatus, the effect of fluid mixing on the enzymatic activity of α-chymotrypsin is evaluated. The findings reported in this article would be valuable to engineers and scientists interested in optimizing and scaling up centrifugal precipitation chromatography.

EXPERIMENTAL

Materials

α-Chymotrypsin from bovine pancreas was purchased from Sigma. Benzoyl-L-tyrosine ethyl ester (BTEE) and sodium phosphate monobasic and sodium phosphate dibasic were also purchased from Sigma. All other chemicals were of analytical grade.

Apparatus

The centrifugal precipitation chromatography unit used was kindly loaned to us by Dr. Ed Chou, Pharma-Tech Research Corp., Baltimore, Maryland, and was similar to the one described by Ito.[6] Briefly, the actual apparatus consists of a separation column made of a pair of flat disks (high-density polyethylene, 13.5 cm in diameter and 1.5 cm in thickness) with a spiral-shaped narrow groove (1.5 mm wide and 2 mm deep) at the periphery.

The dialysis membrane sheet (regenerated cellulose, MWCO 6,000-8,000, Spectrum) is sandwiched between these disks to form two channels, each with a capacity of 5 mL. The column assembly is mounted on the sealless continuous flow centrifuge that allows continuous elution through multiple flow lines of the rotating column without the use of rotary seal.[13]

Enzymatic Activity Assay of α-Chymotrypsin

An assay proposed by Clark and Bailey,[14] and later modified by Cicek and Tuncel,[15] was used in determination of α-chymotrypsin activity. Conceptually, the assay was based on the changes in the absorbance at 258 nm during the hydrolysis of BTEE by α-chymotrypsin. In the assay, a fresh solution of BTEE in borate buffer, pH 7.8, was prepared. 2.5 mL of BTEE solution in borate buffer was then transferred to a silica cuvette. After that, 0.1 mL of α-chymotrypsin solution was added into the cuvette. Immediately after the α-chymotrypsin solution was added, the absorbance at 258 nm of the assay mixture was monitored every 10 seconds until there was no significant change in the absorbance. The apparent BTEE hydrolysis rates or the activity was then calculated by the equation proposed by Clark and Bailey[14] as

$$R = S_0 \frac{\left(\frac{dA}{dt}\right)}{\Delta A}$$

where R is the reaction rate, S_0 is the initial BTEE concentration, dA/dt is the derivative of the absorbance with respect to time during the initial course of the reaction, and ΔA is the differences of the absorbance before and after the reaction is completed.

The specific activity of α-chymotrypsin was then calculated as the ratio of the enzymatic activity of α-chymotrypsin to the concentration of α-chymotrypsin. Bradford assay[16] was used to determine the concentration of α-chymotrypsin present in the solution.

Experimental Procedure

In order to study the effect of shear stress caused by fluid mixing in the separation channel of centrifugal precipitation chromatography on the enzymatic activity of α-chymotrypsin, 1 mL of 5 mg/mL α-chymotrypsin solution in 10 mM sodium phosphate buffer, pH 7.0 (containing 10 mM NaCl), was injected into the separation channel, i.e. the water channel, of centrifugal precipitation chromatography. Sodium phosphate buffer, pH 7.0 (containing 10 mM NaCl), was fed into the separation (water) channel and also into the upper salt channel, which normally contains ammonium sulfate solution.

It should be pointed out, that this experimental design was set up in order to isolate the effect of shear stress caused by fluid mixing on α-chymotrypsin activity. The solution of α-chymotrypsin was subjected to different rotational speeds and different water flow rates in the separation (water) channel.

The eluent of the separation channel was monitored by an UV spectrophotometer at 280 nm and was collected by a fraction collector. Samples containing α-chymotrypsin were pooled, and the concentration and specific activity of these pooled α-chymotrypsin samples were later determined according to the procedures described in the previous section.

In an independent experiment, the effect of precipitation and dissolution was investigated. In this experiment, 70% ammonium sulfate solution was supplied into the upper salt channel while other operating conditions were similar to the set up described above. The rotational speed of 1500 RPM was applied since it was observed that the enzyme was most likely to remain active at this rotation speed. (See below in Result and Discussion).

The flow rate of water fed into the water channel was 0.06 mL/min, since it was shown in the experiment described earlier, that this water flow rate would be less likely to deactivate α-chymotrypsin. The eluent from centrifugal precipitation chromatography was collected, desalted, and analyzed for both α-chymotrypsin enzymatic activity and concentration.

RESULTS AND DISCUSSIONS

The relative enzymatic activity of α-chymotrypsin subjected to different rotational speeds (RPM) and different water flow rates is shown in Figure 1. The relative enzymatic activity of α-chymotrypsin is defined as the specific activity of α-chymotrypsin in the sample compared to that of the standard α-chymotrypsin solution. The Reynold number (Re) was used to represent the effect of water flow rate, while the rotational number (Ro) was used to represent the effect of rotational speed. The Reynold number and rotational number[17] can be defined as

$$\mathrm{Re} = \frac{d_h v}{\upsilon}$$

$$\mathrm{Ro} = \Omega d_h^{\,2} / \upsilon$$

where d_h is equal to 4 times the mean hydraulic radius, v is the fluid velocity, Ω is the angular velocity of the columns, and ν is the kinematic viscosity. According to Ito and Nanbu,[17] as Ro increases, the effect due to the centrifugal force becomes greater.

From Figure 1, it is apparent that the activity of α-chymotrypsin usually decreased when the Reynolds number of water in the separation channel increased, regardless of the rotational number (Ro). The decreases in α-chymotrypsin activity at high Reynolds number are probably the result of more fluid mixing when the fluid in the separation channel flowed faster. More importantly,

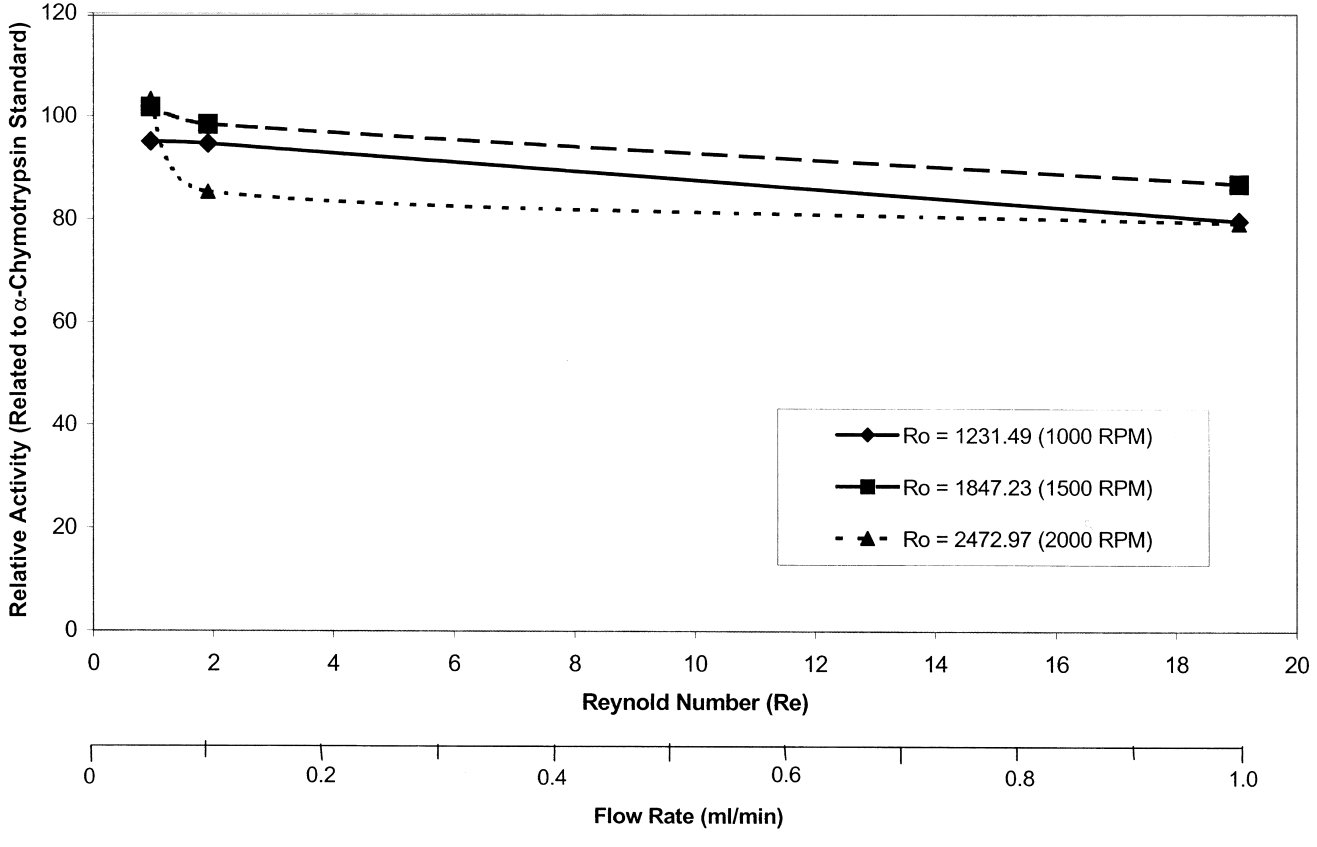

Figure 1. Relative activity of α-chymotrypsin eluted from the separation channel of centrifugal precipitation chromatography as a function of the Reynold number (re) in the separation channel and the rotational number (ro). Sodium phosphate buffer, ph 7.0, was fed into both separation and salt channels.

it is evident in Figure 1 that the effect of fluid mixing in the separation channel on the proteins' activity is minimal at low Reynolds number. Consequently, it is recommended that centrifugal precipitation chromatography should be operated at low Reynolds number.

According to Figure 1, it is also observed that the enzymatic activities of α-chymotrypsin at low Reynold number and rotational number of 1231.49 (1000 RPM), 1847.23 (1500 RPM), and 2472.97 (2000 RPM) are very close to each other; hence, the losses in enzyme activities do not depend on the rotational number at low Reynolds number. This observation implies that one can utilize higher rotational number than the ones used by the authors without any significant losses in protein activity if Reynolds number in the separation (water) channel is low. On the other hand, the losses in enzymatic activities depend on the rotational number at higher Reynold number. In this region, it is found that the rotational number of 1847.24 (1500 RPM) is optimum for operating centrifugal precipitation chromatography.

The complicated changes of secondary flow patterns in the separation channel, for example the formation of double vortices and quadruple vortices, as Reynold number and rotation number vary,[18] are probably responsible for the existence of the optimum rotational number at 1847.24 (1500 RPM). This observation points out that a detailed study of fluid dynamics in the separation channel is needed in order to design a better separation channel, which would not deactivate proteins at high Reynold number.

An independent experiment was performed to investigate the effect of repetitive precipitation and dissolution of α-chymotrypsin in the separation channel. As discussed previously, the 70% saturation ammonium sulfate solution was supplied into the upper salt channel; hence, this set up was similar to the one used to fractionate protein. From the experiment, it was found that 89.03% of α-chymotrypsin remained active.

Although the observations reported here are very important in optimizing and scaling up centrifugal precipitation chromatography, detailed analysis of protein structural changes after passing through the centrifugal precipitation chromatography would undoubtedly provide more information. As a result, future investigations by 2-D NMR will be performed, and the results of the analysis will be reported in subsequent publications.

CONCLUSION

We have shown in this paper, that the effect of fluid mixing in the separation channel of centrifugal precipitation chromatography on the enzymatic activity of α-chymotrypsin is minimal, especially at low Reynolds number. More than 90% of the original enzyme remained active after passing through the separation

channel of centrifugal precipitation chromatography in most cases. Therefore, it is concluded that, most proteins should remain active after being subjected to centrifugal precipitation chromatography when the Reynolds number in the separation channel is low.

Although we have observed some losses in enzymatic activity of α-chymotrypsin after passing through centrifugal precipitation chromatography at higher Reynolds number, it should be noted that all experiments reported here were performed at room temperature without any temperature control. A significant improvement is expected if the experiment is performed at lower temperature, with better channel design and the friendlier precipitating reagents.

ACKNOWLEDGMENT

The authors are indebted to Dr. Ed Chou, Pharma-Tech Research Corp., Baltimore, Maryland, for providing a centrifugal precipitation chromatographic unit.

REFERENCES

1. Englard, S.; Seifter, S. Precipitation Techniques. in *Methods in Enzymology;* Deutscher, M.P., Ed.; Academic Press: San Diego, 1990; Vol. 182, 285–300.
2. Bell, D.J.; Hoare, M.; Dunnill, P. The Formation of Protein Precipitates and Their Centrifugal Recovery. in *Advances in Biochemical Engineering/ Biotechnology*; Fiechter,A., Ed.; Springer-Verlag: New York, 198; Vol. 26, 254–69.
3. Scopes, R.K. *Protein Purification: Principle and Practice*, 3rd Ed.; Springer-Verlag: New York, 1994.
4. Porath, J. Nature **1962**, *196*, 47–48.
5. King, T.P. Biochemistry **1972**, *11*, 367–371.
6. Ito, Y. J. Liq. Chromatogr. **1999**, *22*, 2825–2836.
7. Ito, Y. Anal. Biochem. **2000**, *277*, 143–153.
8. Sookkumnerd, T.; Hsu, J.T.; Ito, Y. AIChE J. **2000**, *46*, 1191–1200.
9. Sookkumnerd, T.; Hsu, J.T.; Ito, Y. J. Liq. Chromatogr., in press.
10. Przybycien, T.D.; Bailey, J.E. Enzyme Microb. Technol. **1989**, *11*, 264–276.
11. Penarrubia, L.; Moreno, J. Biochim. Biophys. Acta **1987**, *916*, 227–235.
12. Beck, C.R. Prediction of Shear Induced Enzyme Activity Losses in Flow Systems, Dissertation, Michigan State University (1979).
13. Ito, Y.; Saudeau, J.; Bowman, R.L. Science **1970**, *189*, 999–1000.
14. Clark, D.S.; Bailey, J.E. Biotech. Bioeng. **1983**, *25*, 1027–1047.

15. Cicek, H.; Tuncel, A. J. Poly. Sci. **1998**,*36*, 543–552.
16. Bradford, M.M. Anal. Biochem. **1976**, *72*, 248–254.
17. Ito, H.; Nanbu, K. J. Basic Eng. **1971**, *93*, 383–394.
18. Speziale, C.G. J. Fluid Mech. **1982**, *122*, 251–271.

Received August 12, 2000 Manuscript 5377
Accepted September 7, 2000