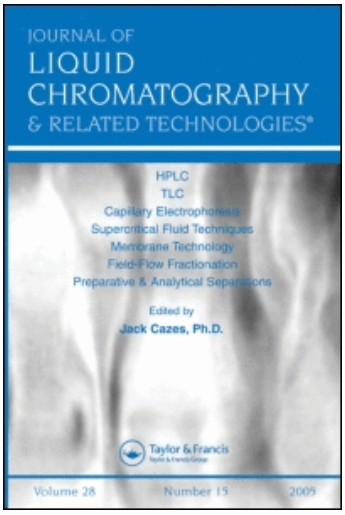

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A. Rasooly[a]; Y. Ito[b]

[a] Division of Microbiological Studies, U. S. Food and Drug Administration, Washington, DC [b] Laboratory of Biophysical Chemistry, National Heart and blood Institute, Bethesda, MD

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TOROIDAL COIL COUNTERCURRENT CHROMATOGRAPHY SEPARATION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN A IN FOOD

Avraham Rasooly,[1] Yoichiro Ito[2,*]

[1] U. S. Food and Drug Administration
Division of Microbiological Studies
Washington, DC

[2] Laboratory of Biophysical Chemistry
National Heart and blood Institute
National Institutes of Health
Bethesda, MD

ABSTRACT

Countercurrent Chromatography (CCC) has the potential to play a major role in food analysis because it permits analysis of crude and complex samples. CCC was evaluated for its ability to separate *S. aureus* enterotoxin A (SEA), which is a common cause of food poisoning. Mushrooms containing native- or heat-denatured SEA were separated by Toroidal Coil CCC and the fractions containing the toxin were analyzed by Western immunoblotting.

Both native SEA, used to spike mushroom samples, and heat-denatured SEA, resulting from *S. aureus* contamination of canned mushrooms, were detectable using CCC.

This method increases the level of sensitivity of Western immunoblotting by at least an order of magnitude by concentrating the sample. Our results suggest that CCC, in combination with a suitable detection method, is a potentially useful method for toxin analysis in food.

INTRODUCTION

Food-borne microbial diseases afflict between 69 and 275 million humans in the US each year.[1] The most common disease is gastroenteritis resulting from food contaminated with enterotoxin A (SEA) produced by the bacterium *Staphylococcus aureus*. SEA is an extremely potent gastrointestinal toxin, with amounts as low as 100 ng being sufficient to cause symptoms of intoxication.[2] Therefore, it is critical that the methods of testing food be able to detect very low levels of SEA.

The most commonly used assay for SEA is an enzyme-linked immunosorbent assay (ELISA), in which monovalent or polyvalent antibodies to SEA are reacted with a food sample. Although the ELISA procedure is simple, sensitive, and rapid, numerous problems have been reported. For example, ELISA detection has been confounded by heat treated samples,[3] false positives due to cross reactivity with food antigens,[4] and naturally occurring peroxides in the food sample.[5]

Western immunoblotting is another immunological detection method, in which antigens are separated by SDS-PAGE, transferred to a membrane, and then reacted with antibodies. The main advantage of Western blotting is that it reveals the molecular weight (or the charge) of the crossreacting antigens, which decreases the probability of false positives. However, only a small sample volume (20 μL - 100 μL) can be evaluated in a Western blot, limiting the ability to detect small amounts of SEA in food samples without additional, time-consuming, purification steps.

Countercurrent Chromatography (CCC) is a chromatography method that utilizes the partitioning of solute between two immiscible solvent phases without a solid support.[6-8] High efficiency of such partioning is achieved by two-phase distribution in a high speed rotating coil. Using a long twisted tubing and an aqueous-aqueous polymer phase system enables partitioning proteins without risk of denaturation.

CCC, unlike other chromatography methods, is capable of handling crude, complex samples and relatively large volumes. *Staphylococcus aureus* is a very common food contaminant found in a wide variety of foods. Thus, SEA testing must be able to accommodate food samples with very different mixtures of proteins,

fats, sugars, nucleic acids, dyes etc. at various pH's and salt concentrations. Since CCC is relatively insensitive to these variables, it may be a good potential tool for food analysis. However, the utility of this method has not yet been recognized.

The purpose of this study was to evaluate the use of Toroidal Coil CCC in combination with Western immunoblotting to overcome the sample size limitation of Western immunoblotting. In this study, mushrooms were chosen as the sample because canned mushrooms were recently implicated in SEA food poisoning in the United States.[9]

Our results demonstrate that CCC extends the level of sensitivity of Western immunoblotting by at least an order of magnitude and suggest that CCC, in combination with an appropriate detection method, has the potential to become an important tool in food analysis.

EXPERIMENTAL

Apparatus

The present studies employed a toroidal coil centrifuge to perform CCC extraction of SEA from mushroom extract. The head of a commercial floor model of the centrifuge (CRT 5000) was modified in such a way that the solvent is passed through the rotating column without the use of a rotary seal device which may produce complications such as leakage and contamination. The detailed design of the apparatus is reported elsewhere.[10,11]

The separation column was prepared from a single piece of 20 m long and 1 mm ID PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) by folding it in two and twisting along its axis to make multiple twisted turns. The twisted tubing was then wound around a spool-shaped support which fit into the centrifuge bowl. The total column capacity was 18 mL.

Reagents

Polyethylene glycol (PEG) 1000 was purchased from Sigma Chemical Co., St. Louis, MO, USA and dibasic potassium phosphate from Mallinckrodt, Paris, KY, USA.

Bacterial Strain and Inoculation

Staphylococcus aureus ATCC 13565 culture was used to inoculate a 113 g can of mushrooms purchased from a local store. The contaminated mushrooms were incubated at 37°C, with shaking, for 5 hours. The contents of the can were then autoclaved at 121°C for 20 min.

Preparation of Two-Phase Solvent System and Sample Solution

The polymer phase system, composed of PEG-1000 and dibasic potassium phosphate, each at 12.5% (w/w) in distilled water, was thoroughly equilibrated in a separatory funnel at room temperature. After two clear layers were formed, the two phases were separated and each stored in a glass bottle.

The mushroom samples were homogenized and an equal weight of distilled water was added. Then, an appropriate amount of PEG-1000 and K_2HPO_4 was added to the above mixture to adjust the phase composition in the sample solution to meet the polymer phase system used for separation. The mixture was centrifuged at 1000g to obtain two clear phases. The partition coefficient (K) of the SEA in this solvent system is about 1; each phase contained SEA at the same concentration. In each separation 0.5 mL of either upper or lower phase was loaded into the column.

CCC Procedure

In each separation, the column was first completely filled with the PEG-rich upper stationary phase. This was followed by injection of sample solution through the sample port. Then the column was rotated at 1200 rpm while the phosphate-rich lower phase was eluted through the column at a flow rate of 0.2 mL/min. The effluent from the outlet of the column was continuously monitored with a UV detector (Uvicord S, LKB Instruments, Stockholm, Sweden) and collected into test tubes using a fraction collector (Ultrorac, LKB Instruments). After 2-3 hours of elution, the centrifuge run was stopped and the column contents were pushed out by N_2 into a graduated cylinder to measure the volume of the stationary phase retained in the column. To reduce the elution volume, the samples were concentrated using an Amicon filter concentrator (MW 3000).

SDS-PAGE and Immunoblotting

Samples were fractionated by 12.5% SDS-PAGE.[12] Samples were heated at 90°C for 2 min immediately before being loaded. The gels were run at 150 V for 2

h, then electroblotted to nitrocellulose membrane (Nitroplus) at 400 mA for 60 min. The membrane was blocked with Tris (10 mM pH 8)-Tween 20 (0.5%)-NaCl (0.5 M) for 20 min and incubated with rabbit anti-SEA (1:300) (Sigma) followed by goat anti-rabbit alkaline phosphatase conjugate (1:1000) (Sigma). A BCIP/NBT solution (Sigma) was used for detection.

Densitometry of Western Blot

The filter was scanned by a Hewlett Packard 4C scanner and the bands were quantitated using NIH Image software.

RESULTS

Toroidal Coil Counterercurrent Chromatography Separation of Native SEA in Mushroom Sample

To evaluate Toroidal Coil CCC for detecting SEA in food, we attempted to separate SEA in mushroom samples. Mushrooms were chosen because several staphylococcal food poisoning outbreaks in the United States have been associated with canned mushrooms.[6]

The canned mushrooms were homogenized and spiked with SEA (15 μg/2mL.). Samples without added SEA served as a control. The sample was applied to a CCC system and separated as described in Experimental.

The solvent system was 12.5% (w/w) PEG 1000 and 12.5% (w/w) K_2HPO_4 in water because the partition coefficient of the toxin was near 1. The lower phase was pumped at a flow rate of 0.2 mL/min and the run was monitored at 280 nm (Figure 1).

To determine if SEA was present in any of the resulting fractions, we used Western immunoblots. The fractions were separated by SDS-PAGE (see Experimental), electrotransfered to a nitrocellulose membrane and incubated with commercial rabbit polyclonal anti-SEA antibodies.

The blot was then washed and incubated with an alkaline-phosphatase conjugated goat anti-rabbit secondary antibody, washed and developed by adding a colorgenic substrate for the alkaline phophatase.

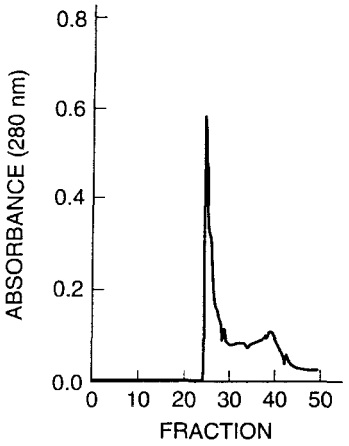

Figure 1. Chromatogram of CCC separation of native SEA added to mushrooms.

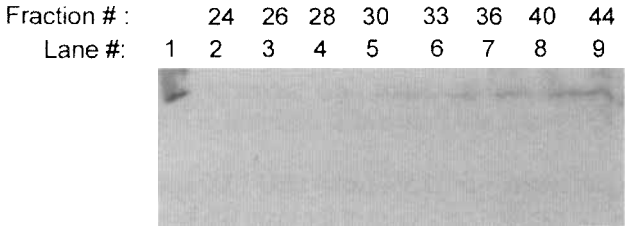

Figure 2. Western immunoblotting analysis of lower phase fractions of CCC separation of native SEA in mushrooms. Lane 1 SEA marker, lanes 2-9 fractions 24-44.

As seen in Figure 2, SEA is detected in fractions 28-44. Although the signal was strong, the sample was spread over 16 fractions (a volume of about 6 mL). No signal was detected in the control sample (data not shown). These results indicate that this CCC protocol is suitable for SEA separation from mushrooms, although the sample needs to be concentrated to reduce the elution volume.

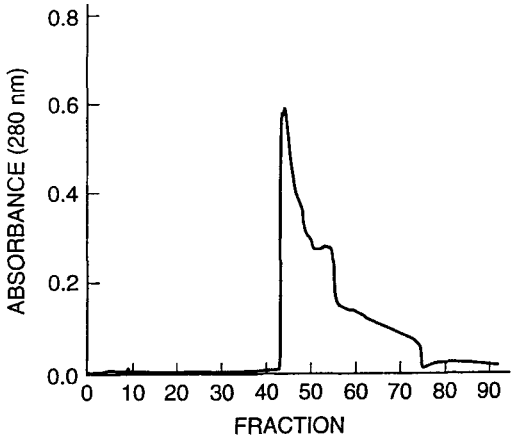

Figure 3. Chromatogram of CCC separation of SEA in heat treated mushrooms.

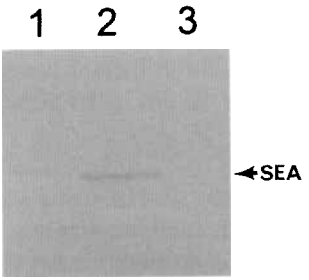

Figure 4. Western immunoblotting analysis of concentrated lower phase fractions of CCC separation of heat treated SEA in mushrooms. Lane 1 crude, unseparated sample of inoculated mushrooms before CCC separation, lane 2 concentrated fractions of inoculated mushrooms, lane 3 concentrated fractions of uninoculated mushrooms.

Toroidal Coil Counterercurrent Chromatography Analysis of SEA-Contaminated, Heat-Treated Mushrooms

ELISA is not always an effective method for determining the presence of SEA in canned food, because the antibodies do not cross-react strongly with heat-treated SEA, resulting in false negatives.[3] To test the ability of CCC to separate SEA from

heat-treated food, we made a sample of heat-treated contaminated mushrooms. Mushrooms were inoculated with *S. aureus* ATCC13565 and incubated for 5 hours (monitoring growth of the bacteria by plating), using conditions that stimulate vigorous bacterial growth. The samples were then autoclaved to simulate the canning process. The control in this experiment was autoclaved uncontaminated mushrooms.

Each sample was then separated by CCC (Figure 3). Before Western blotting, the fractions that were expected to contain SEA, based on the experiments with a spiked sample, were concentrated using an Amicon filter concentrater. Each fraction was then Western blotted to detect SEA.

As seen in Figure 4, no SEA was detected in the control (lane 3). A very intense signal was seen with the concentrated fractions (lane 2) and a weak signal was detected in the crude, unseparated sample before CCC separation (lane 1). The blot was analyzed by densitometry and the signal after CCC separation is 11 times stronger that the signal seen in the starting material.

DISCUSSION

CCC has the ability to separate substances from relatively large volumes of crude and complex samples which makes it a good candidate for use in food analysis. However the potential of this method has not yet been recognized in the area of food testing, so that this is the first report to evaluate the applicability of CCC for food analysis.

Using Western immunoblotting, we have demonstrated here that Toroidal Coil CCC is able to separate both native and heat-denatured SEA from crude samples of mushrooms. This simple method does not require any sample preparation beyond homogenization. Nevertheless, the method is approximately ten times more sensitive than Western blotting alone because very large samples can be applied to the CCC.

Furthermore, CCC separation increases the accuracy of current detection methods. The current methods (ELISA and Western blotting) are both immunological methods, which are prone to false positive results because the antibodies can crossreact with food ingredients. This is a significant problem in crude food samples. CCC separation is based on the physical properties of SEA and, therefore, represents a different separation principle. The combination of CCC separation with Western blotting has the potential of being a powerful and reliable assay system.

Eventually, combining CCC separation with other analytical identification methods, such as mass spectrometry, capillary electrophoresis or HPLC, may lead to non-immunological assays for SEA in food.

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REFERENCES

1. D. L. Archer, F. E. Young, "Contemporary Issues: Disease with a Food Vector," Clin. Microbiol. Rev., **1**, 377-398 (1988).

2. M. I. Evenson, M. W. Hinds, R. S. Bernstein, M. S. Bergdoll, Int. J. Food Microbiol., 7, 311-7, 316 (1988).

3. R. W. Bennett, J. Assos. Off. Agric. Chem., **75**, 6-12 (1992).

4. C. E. Park, M. Akhtar, M. K. Rayman, Appl. Environ. Microbiol., **59**, 2210-2213 (1993).

5. C. E. Park, M. Akhtar, M. K. Rayman, Appl. Environ. Microbiol., **60**, 677-681 (1994).

6. Y. Ito in **Countercurrent Chromatography: Theory and Practice**, N. B. Mandava, Y. Ito eds., Marcel Dekker, New York, 1988, Chapter 3, pp. 79-442.

7. W. D. Conway, **Countercurrent Chromatography: Apparatus, Theory and Applications**, VCH, New York, 1990.

8. Y. Ito, "Countercurrent Chromatography" in **Chromatography,** *V*, E. Heftmann, ed., J. Chromatogr. Library Part A, Elsevier Scientific Publishing Co., Amsterdam, 1992, Chapter 2, pp A69-A107.

9. P. Hardt-English, G. York, R. Stier, P. Cocotas, Food Technol., **44**, 67-77 (1990).

10. Y. Ito, R. L. Bowman, Anal. Biochem., **85**, 614-617 (1978).

9. P. Hardt-English, G. York, R. Stier, P. Cocotas, Food Technol., **44**, 67-77 (1990).

10. Y. Ito, R. L. Bowman, Anal. Biochem., **85**, 614-617 (1978).

11. Y. Ito, J. Suaudeau, R. L. Bowman, Science, **189**, 999-1000 (1975).

12. U. K. Laemmli, Nature, **227**, 680-685 (1970).

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