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RESEARCH NOTE

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An *In vitro* Enzymatic Digestion Method for Estimation of the Acrylamide Contents of Foods

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Abstract In this study, the acrylamide contents of foods were estimated via liquid chromatography (LC)/mass spectrometry (MS)/MS after the food matrix constituents had been degraded with digestive enzymes (i.e., pepsin and pancreatin) and extracted with water. The quantities of acrylamide released from samples of cereal, potato chips, peanuts, and coffee were 62±5.1, 970, 106±20, and 890 ppb, respectively. No acrylamide was detected in samples of soybean curd (*tofu*), fish cake, and ham. Compared to the amounts of acrylamide detected after extraction with water only, we noted no significant differences in the soybean curd, fish cake, potato chip, ham, and coffee samples. However, the quantities of acrylamide released from the cereal and peanut samples were approximately 2-fold larger following pretreatment with the digestive enzymes. This study presents a new *in vitro* enzymatic digestion method which allows for a more accurate estimation of the acrylamide contents of foods.

Keywords: acrylamide, pepsin, pancreatin, enzymatic digestion

Introduction

Since acrylamide was first detected in heated foods in 2002 (1), a variety of analytical methods have been devised to quantify acrylamide contents in food products reliably. The majority of these methods were predicated either on gas chromatography (GC)/mass spectrometry (MS)/MS or liquid chromatography (LC)/MS/MS after extraction with water and clean-up using filtration and ion-exchange columns.

Special attention has been paid to the sample preparation or pretreatment of food samples toward the optimization of acrylamide extraction. The effects associated with particle size, extraction solvent, extraction temperature, extraction time, and defatting have been thoroughly investigated (2). A novel approach to the problem was recently suggested, involving the extraction of acrylamide from the food sample with water, followed by the precipitation of food matrix constituents with acetonitrile (3), or the extraction of the analyte into ethyl acetate after the precipitation of proteins using metal solutions (4).

Daily acrylamide intake in Sweden was estimated to be in the vicinity of 35 μg/day (5). However, *in vivo* measurements of acrylamide levels indicated that the daily intake was about 100 μg/day (6). Based on these results, Eriksson and Karlsson (7) suggested that the amount of bioavailable acrylamide in food products is larger than the quantities estimated in accordance with existing protocols predicated on water extraction.

In this study, a new extraction method was developed for the accurate estimation of the acrylamide contents of food products. Food samples were subjected to acrylamide extraction with water following the degradation of food matrix constituents via digestive enzymes (i.e., pepsin and pancreatin).

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Materials and Methods

Reagents and chemicals The acrylamide, methanol, and water were obtained from Omnisolv, EM Science (Gibbstown, NJ, USA). The formic and acetic acids were acquired from Sigma-Aldrich (St. Louis, MO, USA). The $^{13}C_3$-acrylamide was obtained from CIL (Andover, MA, USA). The pepsin was acquired from Fluka (Buchs, Switzerland). The bile and pancreatin used in this study were supplied by Sigma-Aldrich.

Acrylamide extraction from foods Acrylamide was extracted from food samples via normal water extraction (8) and an *in vitro* enzymatic digestion method. The *in vitro* enzymatic method was developed in accordance with the protocol established in a previous related study (9); one g of sample was measured into a 50 mL centrifuge-tube. Ten mL of HPLC-grade water and 6.67 μL of 0.85% NaCl solution were then added into the tube. After adjusting the pH to 2-2.5 with 0.45 M HCl, 667 μL of pepsin (750 U per mL of 0.2 M HCl) was added and incubated for 2 hr in a shaker (250 rpm and 37°C). After the reaction pH was shifted to 7.0-8.0 with 0.45 M NaOH, 2.67 mL of bile (150 mg per mL of 0.15 M $NaHCO_3$) and 2.67 mL of pancreatin (3,070 U per mL of 0.15 M $NaHCO_3$) were added into the tube, then incubated for 3 hr in a shaker (250 rpm and 37°C). After the pH was adjusted to 7.0-7.5 using HCl, the internal standard ($^{13}C_3$-labelled acrylamide) was added to a concentration of 200 ng/mL. The sample was then incubated for 20 min in a shaker (180 rpm and 20°C), and was centrifuged (896×g and 4°C) for 15 min. A portion of the supernatant was cleaned up and subjected to acrylamide analysis via LC/MS/MS, as has been reported in earlier studies (8, 10, 11).

Results and Discussion

Selection of food samples The water extraction of acrylamide may be affected by food matrix constituents (12-14), and these vary depending on the chemical compositions of foods. Thus, after the foods were classified on the basis of their chemical compositions, food samples representative of each class were selected. Soybean curd (*tofu*) was chosen as a representative of high protein content foods (protein food), cereal as a representative carbohydrate food, sesame oil as a lipid food, fish cake as a protein-carbohydrate food, potato chips as a carbohydrate-lipid food, ham as a protein-lipid food, and peanuts as a protein-carbohydrate-lipid food. Coffee was also selected for use as a model, as it was known to possess a complicated matrix, rendering the analysis of acrylamide levels using normal water extraction methods a difficult proposition (4).

Estimation of acrylamide content The acrylamide concentrations in the representative food samples were determined following pretreatment with the digestive enzymes (the *in vitro* method) (Table 1). The quantities of acrylamide released from the samples of cereal, potato chips, peanuts, and coffee were 62±5.1, 970, 106±20.0, and 890 ppb, respectively. The sizeable standard deviation appears to be attributable to the significant variance in the acrylamide contents of the food samples prepared from different batches, as the standard deviation with regard to repeated measurements of the acrylamide content of samples from a single batch was less than 4.5%. No acrylamide was detected in the samples of soybean curd, fish cake, and ham. Sesame oil was not digested completely by the enzymes under the reaction conditions used in the digestion of the other samples.

No significant differences between the *in vitro* method and the conventional water extraction method were detected with regard to the quantity of acrylamide released from the samples of soybean curd, fish cake, potato chips, ham, and coffee (Table 1). However, the quantities of acrylamide released from the cereal and peanut samples were approximately 2-fold higher following pretreatment with digestive enzymes than after normal water extraction. This shows that the acrylamide contents of some foods may be higher than those determined via normal water extraction.

One of the reasons for the greater efficiency of acrylamide extraction after enzymatic digestion might be that the enzymatic digestion step degrades or destroys the constituents of the food matrix; namely, polypetides, polysaccharides, and lipids. As these complexed matrix structures may result in more space and a greater probability for acrylamide to bind and adsorb to food components (12-14), the degradation of food matrices via digestive enzymes may cause the release of a greater quantity of acrylamide from food samples. During digestion, the extraction solution became transparent (data not shown), which implies that the amount of suspended particles in the solution had been reduced. Thus, the efficiency of acrylamide extraction may be increased as the consequence of enzymatic digestion.

Optimization of the *in vitro* method In order to simplify the *in vitro* method, the amount of acrylamide released over the reaction duration was measured during enzymatic digestion (Fig. 1). The quantity of acrylamide released from the peanut sample increased from 38 to 73 ppb, and then to 119 ppb, after treatment with pepsin and pancreatin, respectively. The quantity of acrylamide detected from the negative control remained at levels of less than 68 ppb throughout the entirety of the experiment. This shows that the quantity of acrylamide released from the peanut sample was increased as the result of digestion with both pepsin and pancreatin.

The reaction pH and temperature employed in the *in vitro* method was identical to those found in the human body. Thus, we surmised that the reaction pH and

Table 1. Effect of sample pretreatment method on the extraction of acrylamide

Sample	Enzymatic digestion (ppb)	Negative control[1)] (ppb)	Water extraction[2)] (ppb)
Soybean curd	ND[3)]	ND	ND
Cereal	62±5.1[4)]	45±12.0	<10
Fish cake	<10	ND	ND
Potato chip	970	910	907±83
Ham	<10	ND	ND
Peanut	106±20.0	68±7.1	39±8.8
Coffee (freeze-dried)	890	880	729±55

[1)]Samples were incubated in the absence of enzymes during enzymatic digestion.
[2)]Samples were pretreated according to the method used in FDA (8).
[3)]Not detected.
[4)]±Values indicate the standard deviation. The standard deviation was determined after analysis of acrylamide contents in a number of food samples produced in different batches. The absence of standard deviation in some values does not indicate 0 of standard deviation; the acrylamide concentration was determined from single measurement.

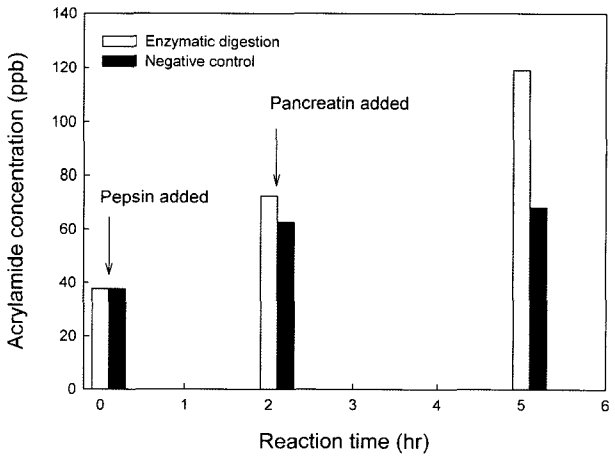

Fig. 1. Effect of enzymatic digestion on acrylamide extraction from peanut sample. Samples were obtained prior to pepsin digestion (t=0), after pepsin digestion (t=2 hr), and after pancreatin digestion (t=5 hr). The negative control indicates that the samples were incubated in the absence of the enzymes.

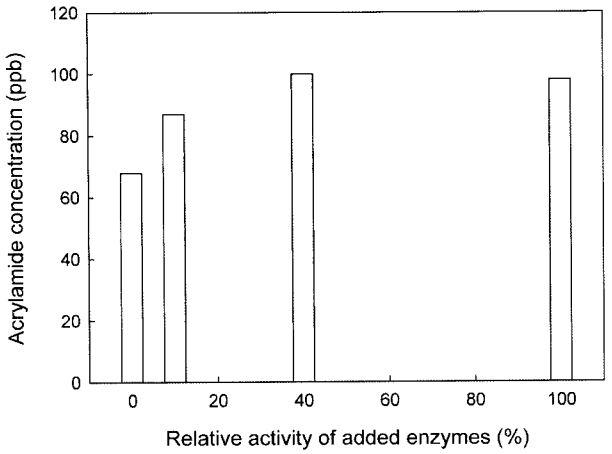

Fig. 2. The effects of the activity of added enzymes on acrylamide extraction from the peanut sample. The sample was pretreated with different activities of digestive enzymes; 100% designates pepsin activity of 500 U, or pancreatin activity of 8,200 U.

temperature utilized would be close to optimal values. The next step in the optimization of the *in vitro* method was focused on the effects of enzyme activity on acrylamide extraction (Fig. 2). When the activity of the added pepsin was reduced from 500 to 200 U, and the pancreatin activity was simultaneously reduced from 8,200 to 3,300 U, the quantity of acrylamide released from the peanut sample did not change to any significant extent. However, when pepsin and pancreatin activities were reduced to 50 and 830 U, respectively, the quantity of released acrylamide decreased to 119 ppb. This indicates that the activity of the added pepsin and pancreatin should be higher than 50 and 830 U, respectively, in order to ensure efficient acrylamide extraction.

The results of the current study indicated that the acrylamide contents of foods can be higher than those observed following normal water extraction; the quantity of acrylamide released from the peanut and cereal samples was approximately 2-fold higher after pretreatment with digestive enzymes. Future studies will focus on the analysis of acrylamide contents in a broader variety of foods, in order to evaluate the relationship existing between the structures of different food matrices and the efficiency of acrylamide extraction.

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