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## Comparison Study Between Crude and Purified L-Asparaginase From *Bacillus* Spp. for Reduction of Acrylamide Level During Fries Processing

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### Abstract

Acrylamide is a toxic chemical that is created when foods are heated; it is also available in foods containing different additives. The purpose of the study was to determine whether *Bacillus* spp. isolates could reduce the concentration of acrylamide in food, as well as to compare the different treatments of crude and pure L-asparaginase produced from the same bacteria in acrylamide reduction in potato slices. Our findings reveal that this bacterium could degrade acrylamide and reduce its concentration. Furthermore, the acrylamide content of potato slices reduced dramatically with increasing enzymatic treatment time, reaching the under detection limit (UDL) after 30 minutes of treatment with 84 U/ml of crude and purified L-asparaginase. In addition, the purified enzyme is more active in removing acrylamide from potato slices than the crude enzyme at all three times. The removal efficiency rises as the enzyme concentration increases. After bleaching the potato slices for 20 minutes at 80 °C with 84 U/ml of pure enzyme, the acrylamide level was totally decreased.

**Keywords:** Acrylamide, potato slices, asparaginase, belching, Acrylic acid

## دراسة مقارنة بين الأسباراجينيز الخام والمنقى من *Bacillus* spp. في خفض مستوى الأكريلاميد في شرائح البطاطا أثناء عملية القلي

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### الخلاصة

يعتبر الأكريلاميد مادة ضارة ويخلق في الأطعمة التي يتم تسخينها ؛ ويوجد أيضًا في الأطعمة ذات الإضافات المختلفة. تهدف الدراسة الى تحديد ما إذا كانت البكتريا العصوية *Bacillus* spp. لها القابلية في خفض الأكريلاميد وبالتالي تقلل من تركيزه في الغذاء ، كذلك مقارنة بين معاملات مختلفة لأنزيم الاسبرجينيز الخام والمنقى والمنتج من نفس البكتيريا في خفض الأكريلاميد في شرائح البطاطا. تظهر النتائج، أن هذه البكتيريا يمكن أن تحلل الأكريلاميد وتسبب انخفاضًا في تركيزه. علاوة على ذلك ، انخفض محتوى الأكريلاميد في شرائح البطاطا بشكل كبير مع زيادة زمن المعاملة الأنزيمية ، حيث وصل إلى تركيز شبه معدوم بعد 30 دقيقة من المعاملة مع 84 وحدة / مليلتر من الاسبرجينيز(الخام والمنقى) ، كما اظهر الانزيم المنقى فعالية اكبر من الانزيم الخام في خفض الاكريلاميد في اوقات المعاملة الثلاثة. كذلك بينت النتائج ان فعالية الانزيم المنقى اكبر في إزالة الأكريلاميد في شرائح البطاطا مقارنة مع الإنزيم الخام ، كما ازدادت فعالية الإزالة مع زيادة تركيز

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الإنزيم. تلاشي محتوى الأكريلاميد تمامًا عند سلق شرائح البطاطا لمدة 20 دقيقة بدرجة حرارة 80 °م بعد المعاملة بـ 84 وحدة / مل من الإنزيم المنقى.

## 1. Introduction

Life forms, including animals, plants, and microscopic organisms, all include L-asparaginases (L-asparagine amidohydrolase; EC 3.5.1.1), which catalyze the hydrolysis of L-asparagine on the amide side to aspartic acid and ammonia [1]. The characteristics of L-asparaginases from diverse bacteria have currently been found [2], including those from *Erwinia*, *Pseudomonas* spp., *Bacillus*, *Aspergillus*, and *Escherichia coli* [3, 4]. For more than 40 years, L-asparaginase has been considered an essential component of a multidrug chemotherapy regimen often used to treat childhood acute lymphoblastic leukemia, Hodgkin's lymphoma, lymphosarcoma, and melanosarcoma [5].

Due to the amount of ammonia produced by the enzymatic reaction and its association with the level of L-asparagine in the patient's blood, L-asparaginases have also been utilized as diagnostic biosensors for L-asparagine [6]. L-asparaginases have been well-described as inhibitors of acrylamide creation in heated food in addition to their clinical usage [7]. Food products that have been cooked or fried contain acrylamide, which is recognized as neurotoxic and carcinogenic [8]. Consuming foods containing high levels of acrylamide is frequently linked to toxicity, which raises the risk of cancer. As a category 2A carcinogen, it is regarded as "probably carcinogenic to humans" [9]. Various starches, including crisps and French fries, have been found to contain acrylamide in amounts ranging from 200 to 1800 g/kg [10]. The potato has been made into a common all-purpose food and is a significant crop that is grown in fields all over the world. However, due to the high content of free L-asparagine and reduced sugars in potatoes, potato-based food products are among those with the highest acrylamide levels [11]. According to earlier studies on acrylamide levels in Chinese food, acrylamide content varies depending on raw material sources and distinct commercial potato products' processing methods. Compared to other products, this content is also relatively high [12].

In the previous study, French fries were chosen as the dietary matrix for L-asparaginase use since they are a representative of potato products. In order to reduce the formation of acrylamide in food products, a variety of techniques have been used [13]. The most effective method to lower the amount of acrylamide in food products that have undergone heat processing is the L-asparaginase enzyme [14]. Enzymatic treatment has been suggested as a simple and effective way to eliminate acrylamide in foods with no impact on the end product's sensory or nutritional properties [15]. The objective of this study was to investigate the hypothesis that *Bacillus* spp. is able to degrade acrylamide and reduce its environmental concentration. A number of experiments were conducted with the aim of verifying whether *Bacillus* spp. may decrease acrylamide concentration. In addition, a comparative study was conducted to reduce acrylamide in potato chips treated with crude and purified asparaginase production from *Bacillus* spp.

## 2. Material and Methods

### 2.1 Media and Chemicals

Nutrient agar, acrylamide, ZnSO<sub>4</sub>, Carrez I and II reagents, and all other major chemical reagents were obtained from India's Hi-Media and Sigma-Aldrich.

### 2.2 Preparation of bacteria

*Bacillus* spp. isolates were obtained from the Biotechnology Department of the Faculty of Science at the University of Baghdad, Iraq. It was previously identified with a biochemical test and a Vitek Compact 2 system, then maintained on nutrient agar medium and refrigerated at 4 °C for the screening experiment. Before each experiment, the bacteria were grown in nutrient broth at 37 °C for 24 hours to establish fresh growth.

### 2.3 Preparation of the acrylamide stock solution

To make "stock" solutions of acrylamide with concentrations of 75, 150, 300, and 1000 mg/L, a concentrated aqueous solution of acrylamide (1 g/L) was made and, if needed, diluted with sterile distilled water. Before the experiments, the solutions were stored in the dark at a cold (4 °C) temperature [16].

### 2.4 The evaluation of acrylamide degradation by *Bacillus* spp.

Initially, it is crucial to monitor the isolate's capacity to degrade acrylamide and its efficacy in reducing the concentration of acrylamide in the medium. In order to do this, one ml of the appropriate acrylamide "stock" solution was added to 8 ml of nutrient broth, which resulted in final concentrations of 7.5, 15, 30, or 100 µg/mL. Following this, samples were inoculated with 1 ml of *Bacillus* isolate (previously prepared). The samples were centrifuged at 10,000 rpm for 5 minutes at the start (0 h), 24 h, and 48 h of incubation at 37 °C, and the supernatant has been collected for the analysis of the content of acrylamide by model HPLC chromatography (SYKAM) in Germany. Acetonitril: D.W. (60:40 V/V) served as the mobile phase, and the separation column was a C18-OSD (25 cm \* 4.6 mm) with a flow rate of 1 mL/min and a fluorescence detector operating at Ex = 217 nm and Em = 334 nm. For each of the three time intervals (0 h, 24 h, and 48 h), separate sets of experimental variants were created. The positive controls lacked microorganisms and simply included nutrient broth and a suitable acrylamide stock solution (concentration 7.5–100 µg/mL). Negative controls consisted of tubes containing nutrient broth inoculated with bacterial suspension (as described above) but without the addition of acrylamide (instead of 1 mL of sterile water). To establish that acrylamide degradation was dependent on microorganisms, the acrylamide concentration in test samples was compared to that in the controls (bacteria-free samples). Two repetitions of the experiment were carried out. Controls confirmed that the samples had no detectable levels of acrylamide [16].

### 2.5 Production, purification and characterization of L-asparaginase

Previously, *Bacillus* spp. was utilized to produce crude L-asparaginase through submerged fermentation in an optimal medium. The preferred medium for production was medium 2, which consisted of glucose (2 g/L), KH<sub>2</sub>PO<sub>4</sub> (1.52 g/L), KCl (0.52 g/L), MgSO<sub>4</sub> (0.52 g/L), FeSO<sub>4</sub> (0.03 g/L), ZnSO<sub>4</sub> (0.03 g/L), and CuSO<sub>4</sub> (0.05 g/L). The optimal conditions for production were achieved using fructose as the carbon source at a pH of 6 and a temperature of 40 °C. After 24 hours of incubation, the specific activity of the produced L-asparaginase was determined to be 260 U/mg protein. The enzyme was purified through gel filtration chromatography using Sephadex G-150. The final purification fold was 2.5, and the yield of the enzyme was 93.7%. The optimal pH for L-asparaginase activity is 7.0, while its stability is highest at pH 8.0. The enzyme exhibited both activity and stability at a temperature of 37 °C. The enzymatic activity was observed to decrease upon incubation with certain metal ions at concentrations of 1 and 5 mM.

### 2.6 Assay for L-asparaginase

Al-Dulimi [17] says that the Nesslerization method, which is based on the conversion of L-asparagine to ammonia and L-aspartate and has a limit of 625 nm for absorption, was changed in small ways. After adding 1 ml of crude L-ASNase, 1 ml of 200 mM L-asparagine in potassium phosphate buffer (0.05 M, pH 8.0) was added. The tube was kept at 37 °C for 30 minutes. Following the incubation, 1 ml of 1.5 M trichloroacetic acid was added to the solution mixture to stop the reaction. The mixture was centrifuged at 8000 rpm for 10 minutes. The direct Nesslerization method was used to measure the L-ASNase activity in the enzyme supernatant for each sample. This was done by vigorously shaking together 1 mL of Nessler's reagent and 1 mL of enzyme supernatant. The mixture was then kept at 37 °C in an incubator for 30 minutes. At 625 nm, the optical density was determined. To prepare the blank, 1 mL of Nessler's reagent was added to 1 mL of the above reaction mixture, except that 1 mL of trichloroacetic acid was added before adding 1 mL of crude enzyme. The amount of enzyme that, under experimental conditions, releases 1  $\mu$ mole of ammonia per minute was designated as one unit of L-ASNase. Based on the Bradford method, protein content was determined [18].

## 2.7 Preparation of fries

Raw potatoes were peeled and sliced into 2-mm-thick slices using a modified version of [19]'s procedure. Additionally, ultrapure water was used to rinse away the starch granules that had adhered to the potato surface.

### 2.7.1 Reducing acrylamide formation in fried potato chips by the use of various treatments and combinations

To reduce the formation of acrylamide, potato chips were subjected to a different set of experiments. The potato slices in set (A) were blanched for 5 and 20 minutes, respectively, at 80 °C. In set (B), potato slices were pretreated separately with crude and purified L-asparaginase (84 U/ml) for 5, 15, and 30 min at 37 °C. In Set (C), potato slices were pretreated with 84, 42, and 21 U/ml of crude enzyme and purified separately for 30 min. In set (D), potato slices were treated with 84 U/mL of crude and purified L-asparaginase separately for 30 min, blanched at 80 °C for 20 min, and then washed thoroughly with ultrapure water. The control was potato slices without any treatment. In each case, including the control, potato slices were dried in an oven (60 °C) for 20 minutes before being fried in oil heated to 180 °C for 5 minutes. All tests were performed in triplicate [20, 21].

### 2.7.2 Extraction of acrylamide from the chips and measured the quantification of its content by high performance liquid chromatography (HPLC).

According to Jia [22], potato chips' acrylamide content was extracted. To start, a blender homogenizer was used to grind the chips into a fine powder. 10 mL of n-hexane were added to a 50 mL glass tube that had been filled with one gram of the sample. Violent shaking was used to remove the samples' long-chain fatty acids. Following the solutions, 10 mL of distilled water, 500 L of Carrez I (15 g potassium hexacyanoferrate/100 mL of water), and 500 L of Carrez II (30 g ZnSO<sub>4</sub>/100 mL of water) were added. For the extraction of the acrylamide, the sample was combined for 30 minutes in a stirred water bath. The homogenates were then centrifuged for 20 minutes at 4 °C using a 10,000 rpm centrifugation. The precipitate was removed, and the supernatant was used to measure the concentration of acrylamide using HPLC chromatography (SYKAM), a German type. Acetonitril: D.W. (60:40 V/V) served as the mobile phase, and the separation column was a C18-OSD (25 cm \* 4.6 mm) with a flow rate of 1 mL/min and a fluorescence detector operating at Ex = 217 nm and Em = 334 nm.

### 3. Results and Discussion

#### 3.1 The evaluation of acrylamide degradation by *Bacillus* spp.

Acrylamide is neurotoxic, carcinogenic, genotoxic, and teratogenic. Its common usage in different industrial processes negatively impacts the environment [23]. The change in acrylamide concentration produced by *Bacillus* spp. based on the initial acrylamide dose and incubation time is detailed in these results. The tested isolate was found to be capable of reducing the acrylamide levels in the environment from 7.5 to 100 µg/mL, which was confirmed by HPLC (Table 1). The acrylamide concentration reached the limit of detection at 7.5 and 15 µg/mL after 48 hours and decreased to 12.2 and 26.1 µg/mL at 30 and 100 µg/mL, respectively.

**Table 1:** The degradation of various concentrations of acrylamide during the incubation of *Bacillus* spp. in nutrient broth medium

Time (h)	Acrylamide's initial Concentration (µg/mL)	Acrylamide's remaining concentration (µg/mL)
0		
24	7.5	2.4
48		UDL
0		
24	15	10
48		.UDL
0		
24	30	17.9
48		12.2
0		
24	100	66.4
48		26.1

**\*UDL= Under Detection Limited**

The first step in biodegrading polyacrylamide is for amidase to break down polyacrylamide into ammonia and polyacrylate. Bacteria then use the ammonia that has been produced as a source of nitrogen. Compared to the amide moieties, the carbon backbone of the polyacrylate is more resistant to biodegradation. However, findings suggest that using polyacrylamide and polyacrylate as carbon sources can promote microbial development. It has been demonstrated that certain aerobic bacteria may catabolize polyacrylamide and polyacrylate when isolated from soil and surroundings that include these materials [24]. According to [25], acrylamide can be broken down into ammonia (a source of nitrogen) and acrylic acid (a source of carbon) under different aerobic and anaerobic conditions. Ammonia can then be turned into hydroxypropionate and oxidized to CO<sub>2</sub>, acetate, propionate, lactate, or acrylyl-CoA.

#### 3.2 Treatment of French fries with various procedures and combinations to reduce the formation of acrylamide after frying

Researchers say that the Maillard reaction is the main way that acrylamide is made in foods [26]. This reaction happens when food components, such as amino acids and reducing carbohydrates, come together during heat treatment. The initial step in this reaction is the formation of the Schiff-base intermediate. This Schiff base can be hydrolyzed further into 3-aminopropionamide, a precursor to acrylamide, or it can be converted directly to acrylamide via an amide group removal method [27]. Because the amino acid L-asparagine is thought to be the main source of acrylamide in products made from potatoes, it is important to see how well the enzyme reduces this component [28]. Furthermore, a high amount of this amino acid in potatoes may be linked to increased acrylamide formation. According to the Swedish National Food Administration (SNFA) in 2002, acrylamide was found in foods high in carbohydrates that had been heated [29]. Between 0.3 and 1.9 g/kg orally have been estimated

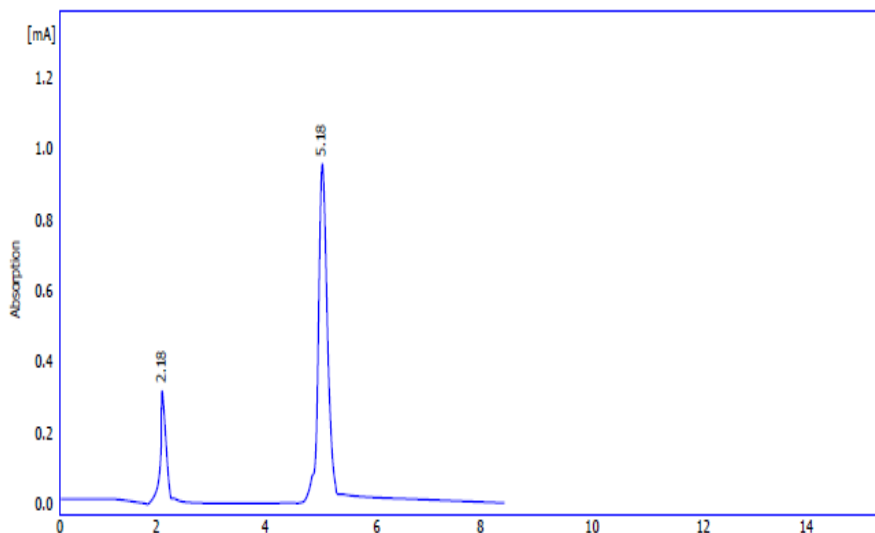
as the dietary intake of acrylamide [30]. A typical industrial potato chip process includes a unit operation called blanching, which is normally carried out in hot water (65–80 °C) for 10–30 minutes [7].

In this study, the amount of acrylamide in French fries that were not blanched or treated with L-asparaginase (the control sample) was found to be 181.1 g/ml (Figure 1). The results shown in set (A) were: The acrylamide level could be reduced by blanching at 80 °C to 45.59 and 90.85 µg/ml for 5 and 20 minutes, respectively (Figures 2A, 2B). Because some acrylamide precursors were able to escape during blanching, the formation of acrylamide was slightly reduced. The operation temperature and blanching time have the greatest effects on acrylamide reduction [31]. The key element in reducing acrylamide is bleach temperature, and short-term high-temperature bleach is more effective than lower-temperature bleach. However, compared to products not blanched during the frying process, blanching at higher temperatures (97 °C) might result in undesirable sensory attributes, such as increased oil levels and worse textural characteristics [32]. Obviously, normal high-temperature blanching is detrimental for mesophilic L-asparaginase treatment since it easily inactivates the enzyme. As a result, an extra step of running at a lower temperature after blanching should be undertaken in this procedure to adequately demonstrate the hydrolysis activity of L-asparagine [33]. Similar results were observed in group (B), where the amount of acrylamide significantly decreased with increasing treatment time, reaching 40.58 and 3.25 µg/ml, respectively, after 5 minutes of treatment with 84 U/ml of crude and purified L-asparaginase (Figure 3 (A, B)) and UDL after 15 and 30 min of treatment with 84 U/ml of crude and purified enzyme (Figure 4 (A, B) and Figure 5 (A, B), respectively). As a result, especially with a longer duration, pretreatment with pure L-asparaginase had a substantial effect on acrylamide reduction.

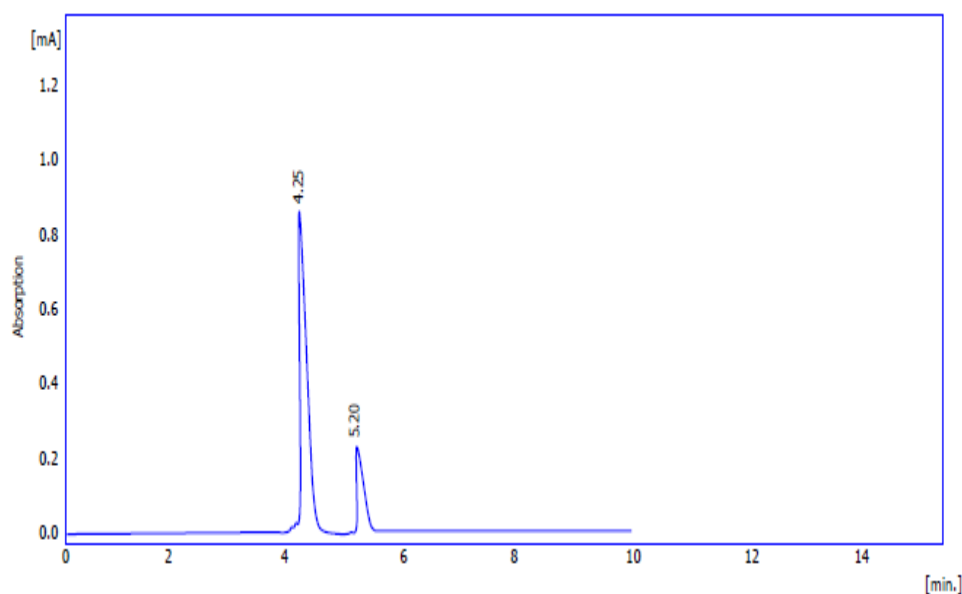
This good result can be explained by the fact that L-asparaginase broke down L-asparagine, which is a key precursor to acrylamide in potato chips, as the treatment time went on, showing how useful the enzyme is in the food industry [21]. L-asparaginase from *Bacillus subtilis* B11-06 reduced the amount of acrylamide in potato samples from 2.53 mg/kg to 0.46 mg/kg [22]. *Bacillus licheniformis* RAM-8 L-asparaginase cut the amount of acrylamide in potato strips by 80% [34], and *L. coli* L-asparaginase stopped almost all of the acrylamide from forming in a snack made with microwaved potatoes [35]. In set (C), the results showed that purified L-asparaginase was more effective than the crude enzyme at removing acrylamide from potato slices, and the removal rate went up as the enzyme concentration went up. Residual acrylamide concentration, represented by UDL, was reached when treated with 84 and 42 U/ml and 6.25 µg/ml at 21 U/ml of purified enzyme for 30 min, respectively (Figures 5 (B) and 6. (A, B)) and reached UDL of 12.56 and 33.58 µg/ml at 84, 42, and 21 U/ml of crude enzyme for 30 min, respectively (Figures 5 (A) and Figure 7. (A, B)).

As the L-asparaginase dose and incubation period were increased, the acrylamide level decreased [33]. Chi and his colleagues [21] found that acrylamide was gone after a 30-minute treatment at 37 °C with 40 U/ml of L-asparaginase from *Mycobacterium gordonae*. The fact that L-ASNase hydrolyzes L-asparagine (an essential acrylamide precursor) in potato crisps demonstrates the enzyme's efficacy in the food industry. Set (D) results showed that the acrylamide content was completely stopped when bleached for 20 minutes at 80 °C after being treated separately with 84 U/ml of crude enzyme and purified enzyme (Figures 8 (A) and (B)). Zuo and his coworker [33] found that treating bleached fibers with L-asparaginase from *Thermococcus zilligii* cut the amount of acrylamide made by a lot. By treatment with 10 U/mL of L-asparaginase during bleaching at 80 °C for 2, 4, 8, and 15 min, the acrylamide level was reduced from 1592 µg/kg to 353, 311, 275, and 232 µg/kg, respectively. The use of L-

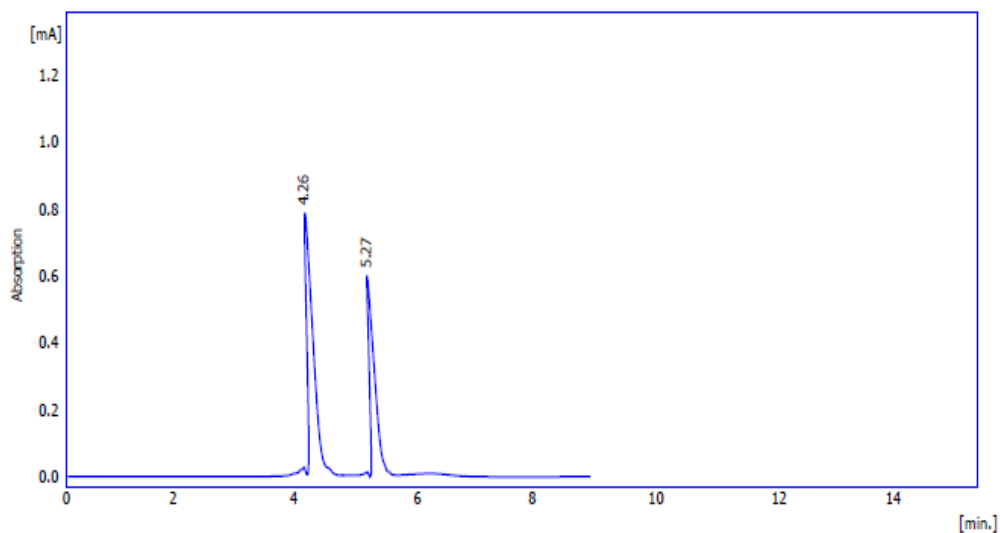
asparaginase presents a relatively new, promising, and excellent technique for reducing acrylamide levels in foods. To maximize the overall effect of the enzymatic method, it may be necessary to adapt the pre- and post-treatment procedures, for example, by reducing the size of the starting material and blanching before enzymatic treatment and modifying the process conditions after enzymatic treatment, for example, by changing the firing temperature and pH [36].



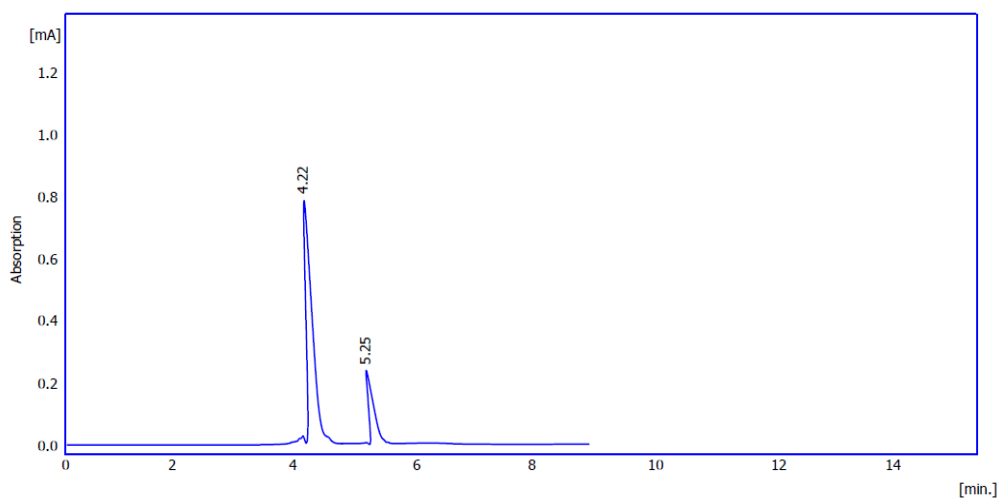
**Figure 1:** Chromatogram of Acrylamide concentration in control sample



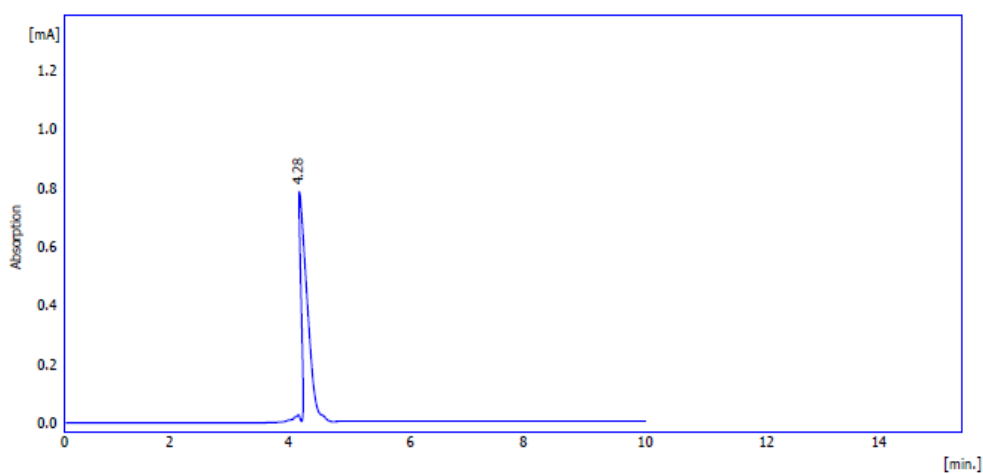
**Figure 2A:** Chromatogram of Acrylamide concentration in blanched potato slices at 80 °C for 5



**Figure 2B:** Chromatogram of Acrylamide Concentration in Blanched Potato Slices at 80 °C for 20

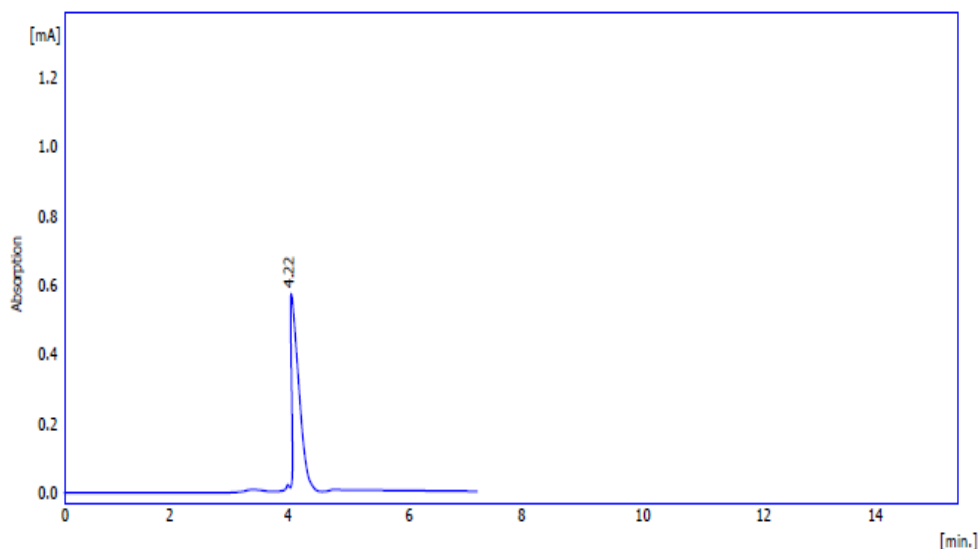


**Figure 3A:** Chromatogram of acrylamide concentration in potato slices pretreated with crude L-asparaginase (84 U/ ml) for 5 min

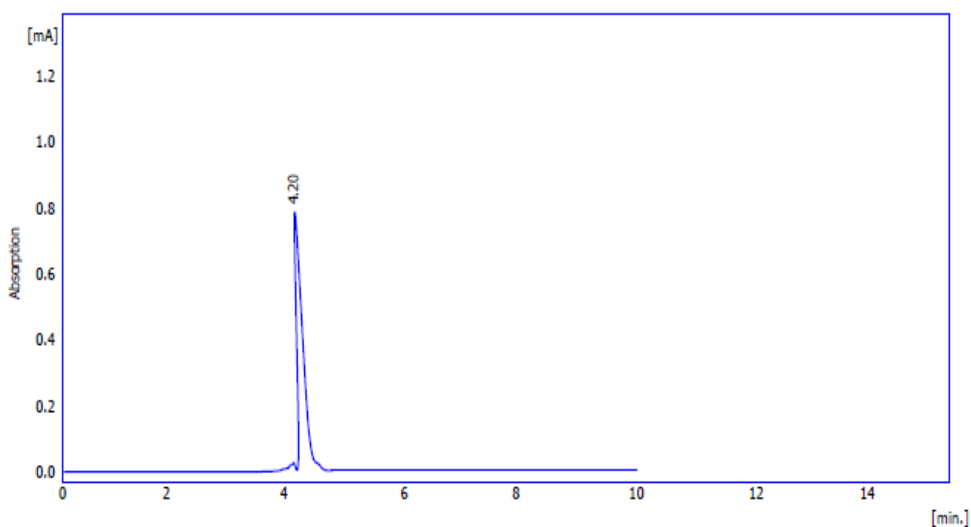


**Figure 4A:** Chromatogram of acrylamide concentration in potato slices pretreated with crude L-asparaginase (84 U/ ml) for 15 min

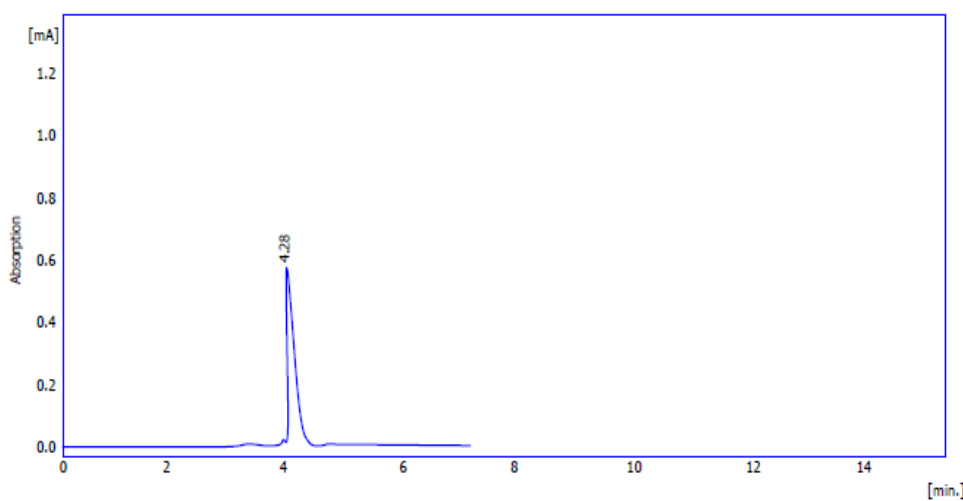




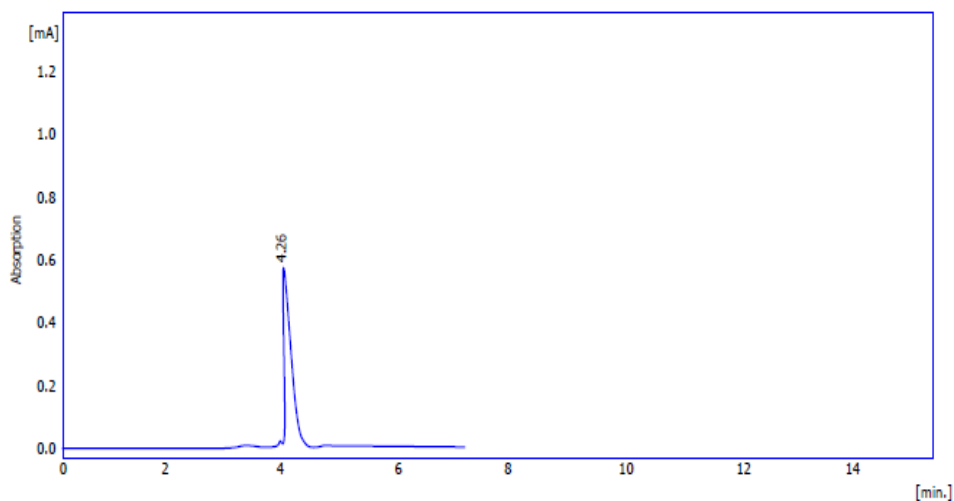
**Figure 4B:** Chromatogram of acrylamide concentration in potato slices pretreated with purified L-asparaginase (84 U/ ml) for 15 min



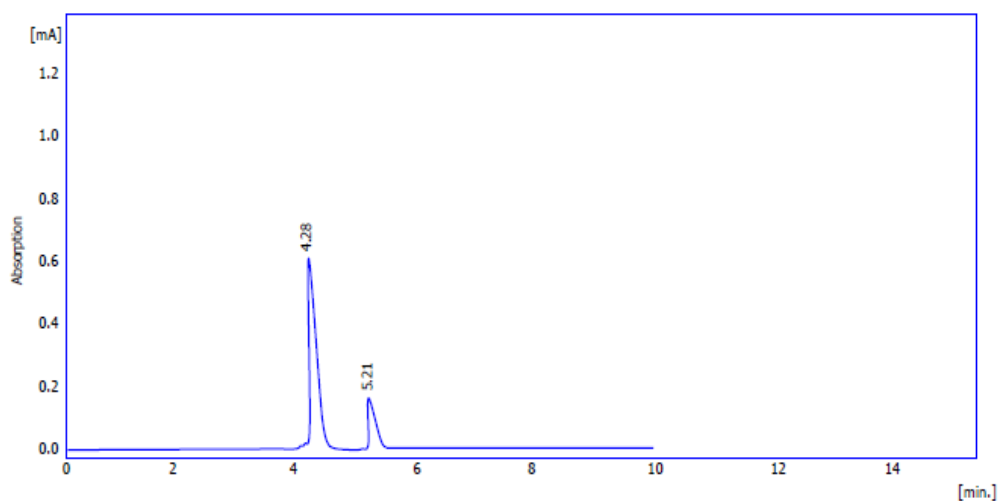
**Figure 5A:** Chromatogram of acrylamide concentration in potato slices pretreated with crude L-asparaginase (84 U/ ml) for 30 min.



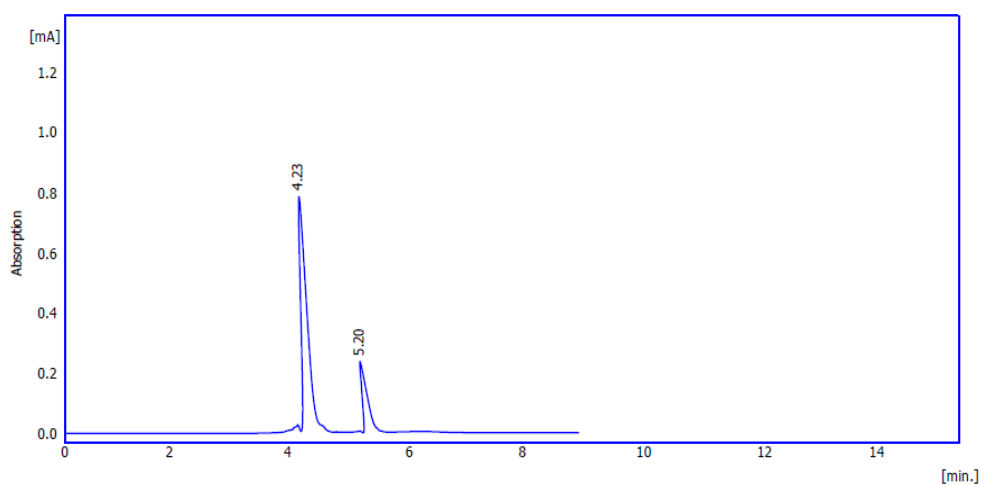
**Figure 5B:** Chromatogram of acrylamide concentration in potato slices pretreated with purified L-asparaginase (84 U/ml) for 30 min.



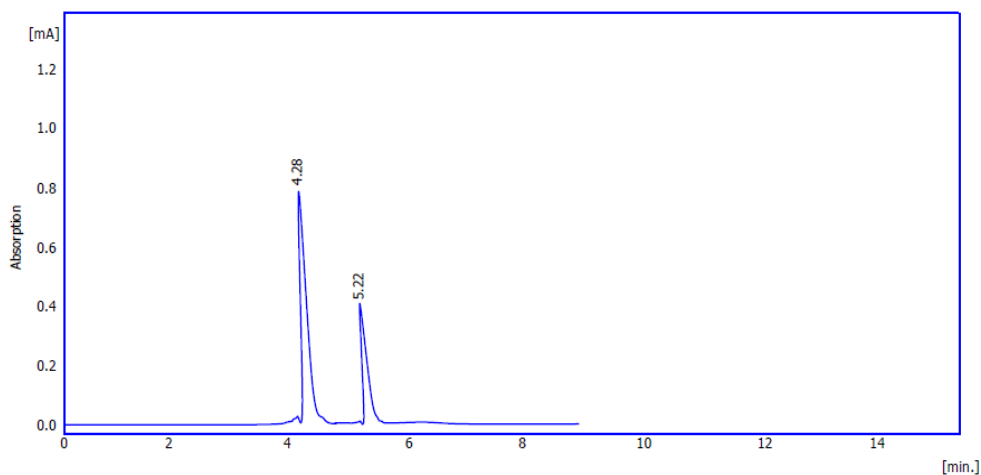
**Figure 6A:** Chromatogram of acrylamide concentration in potato slices pretreated with purified L-asparaginase (42 U/ml) for 30 min.



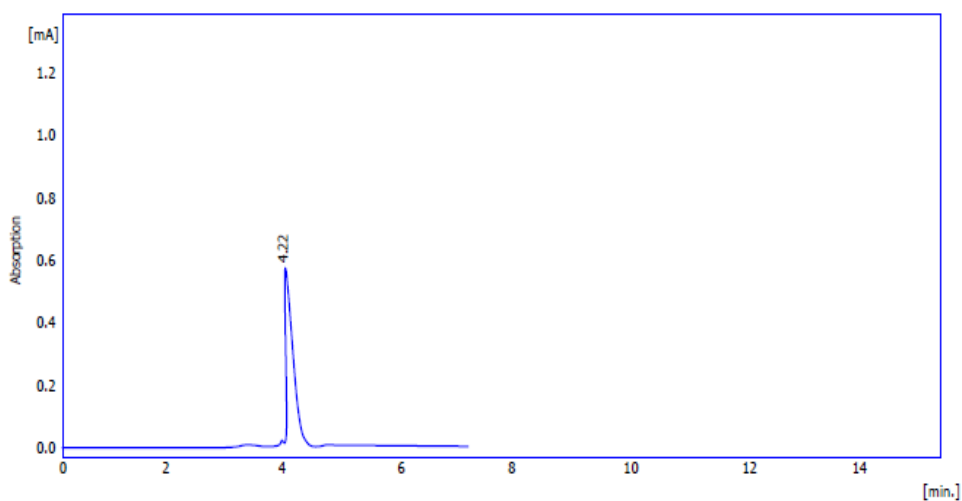
**Figure 6B:** Chromatogram of acrylamide concentration in potato slices pretreated with purified L-asparaginase (21 U/ml) for 30 mi



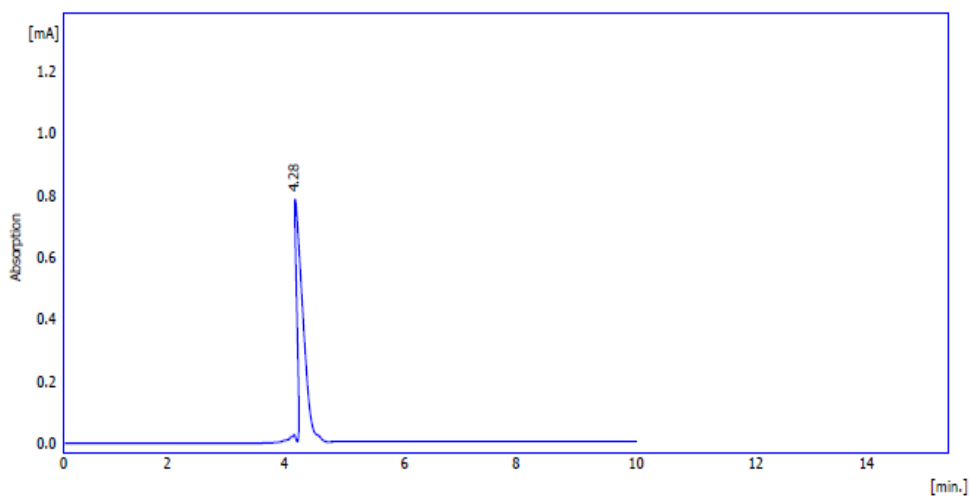
**Figure 7A:** Chromatogram of acrylamide concentration in potato slices pretreated with crude L-asparaginase (42 U/ml) for 30 min.



**Figure 7B:** Chromatogram of acrylamide concentration in potato slices pretreated with crude L-asparaginase (21 U/ ml) for 30 min



**Figure 8A:** Chromatogram of acrylamide concentration in potato slices pretreated with (84 U/ml) of purified L-asparaginase for 30 min, then blanched



**Figure 8B:** Chromatogram of acrylamide concentration in potato slices pretreated with (84 U/ml) of crude L-asparaginase for 30 min, then blanched at

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