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Research Article

Construction and characterization of murine single-chain variable fragment (*MuscFv*) antibody against acrylamide in coffee

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Abstract

Ingredients of food, especially sugar and starch at high-temperature cooking processes could lead to the formation of acrylamide (AA). This chemical is a harmful carcinogen, a neurotoxicant, a reproductive toxicant, and a carcinogen in animal species. However, the detection of acrylamide contamination in food goes unnoticed. In this work, the mouse monoclonal antibody in the form of a single chain variable fragment (*scFv*) specific to acrylamide was selected from the murine *scfv* (*muscfv*) phage-displayed library. Acrylamide (AA) was used as an antigen for bio-panning. The murine single-chain variable fragment (*MuscFv*) antibody specific to acrylamide in coffee was successfully constructed, which was determined by ELISA and HPLC. Currently, this is the first study, which describes the selection of antibodies against acrylamide from the *muscfv* phage-displayed library and could be used as a tool for the detection of acrylamide in coffee.

Introduction

Acrylamide (AA), an unsaturated-amide small molecule, is a by-product of food heating processes (due to the Maillard reaction) that are commonly present in cooked foods. This agent was known to be toxic to humans [1,2] It is rapidly absorbed after ingestion and distributed in many organs such as the thymus, liver, heart, brain, and kidneys [3,4]. Due to its genotoxicity and carcinogenicity [5], acrylamide was classified as a Group 2A carcinogen by the International Agency for Research on Cancer (IARC, 1994) and a Category 2 carcinogen and Category 2 mutagen by the European Union.

Acrylamide contamination in food is first reported by the Swedish National Food Administration using the LC/MS/MS method. The most common foods often found containing acrylamide are potato chips, french fries, baked bread, chocolate, and coffee [6]. Many researchers have confirmed the presence of acrylamide in different processed foods. For Thai foods, acrylamide has been found in curries, commercial and conventional snacks, instant noodles as well as coffee that contain starch and fat as the major components and are cooked at high temperatures [7]. In addition, as reported in the EFSA's scientific opinion on AA in food, the exposure data reported that coffee was one of the sources of this toxicant in adult diets. Several methods for acrylamide detection are reported in the literature, including high-performance liquid chromatography (HPLC) [8], gas chromatography [9], gas chromatography

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coupled with mass spectrometry (GC-MS) [10], liquid chromatography coupled with mass spectrometry (LC-MS/MS) [11,12], immunoassay [13] electro-chemical- biosensors [14] , fluorescent method [4] and quartz microbalance sensors [15] . Most of the methods need advanced equipment. This is an important issue because acrylamide is suspected to cause cancer in humans and there is no concern about acrylamide contaminated [16]. Thus, a device for detecting acrylamide in foods is urgently required. In this study, the *MuscFv* specific to acrylamide was selected and tested against acrylamide using enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Antibodies and reagent

Mouse anti-M13, goat anti-mouse HRP-conjugated polyclonal antibody (GE Healthcare, Dako, Denmark, EU), Mouse monoclonal anti-C-Myc (BioLegend, San Diego, USA), T4 DNA Ligase, *Nco*I and *Not*I restriction enzymes (Thermo Scientific, USA), Acrylamide standard (AA) and Isopropyl-β-D-thio-galacto-pyranoside (IPTG) (Sigma-Aldrich, USA) pSEX81, pOPE101 phagemid vector (Progen Biotechnik GmbH, Heidelberg, Germany) were used in this study.

Selection of murine *scfv* specific to acrylamide

The murine single chain variable fragment (*muscfv*) phage display library was kindly provided by Asst. Prof. Dr. Jeeraphong Thanongsaksrikul from the Faculty of Allied Health Sciences, Thammasat University, Thailand [17]. The AA was used as the antigen to select single-chain fragments (*scfv*) from the library by bio-panning [17]. After bio-panning, XL-1 Blue *E.Coli* colonies containing recombinant *muscfv*-pSEX81 surface expression phagemid vector (Progen Biotechnik GmbH, Heidelberg, Germany) were tested by direct colony PCR using pelB Primer 5>-ATACCTATTGCCTAC GGCAGC-3> and gIII Primer 5>-TAGCATTCCACAG ACAGCCC-3>. The *muscfv* display phage particles were rescued from each individual *E.coli* clone by co-infecting the bacteria with M13KO7 helper phages (GE Healthcare Life Sciences, Denmark, EU). The titers of the rescued phages were determined and normalized for acrylamide-specific binding testing by indirect ELISA. Acrylamide and BSA were used as antigens and blank, respectively. The *E.coli* clone containing *muscfv* that gave optical density at 450 nm. two times higher than blank were selected and subcloned into the pOPE101- plasmid (Progen Biotechnik GmbH, Heidelberg, Germany) for recombinant murine *scfv* production.

Small-scale *MuscFv* expression

The XL-1 Blue *E.coli* containing *muscfv*-pOPE101 phagemid vector was grown in 10 mL LB-broth supplemented with 100 µg/mL ampicillin in a 37 °C shaker until $OD_{600\,nm}$ = 0.6 Isopropyl-β-D-thio-galacto-pyranoside (IPTG) (Sigma-Aldrich, USA) was added to a final concentration of 1 mM. Cells were harvested 3 h later, and centrifuged at 10,000 rpm for 20 min at 4 °C. The cells were lysed by sonication in a lysis buffer.

Binding analysis by indirect ELISA

The *E.coli* lysate was tested for binding to acrylamide standard (AA) according to the protocols described [17]. Briefly, acrylamide standard (5 µg) and BSA in 100 µl coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) were immobilized on ELISA wells and kept at 37 °C until dry. After washing with PBST, all wells were blocked with 3% BSA in PBS and kept at 37°C for 1 h. Next, the standardized *E.coli* lysate was individually added into wells and kept at room temperature for 1 h. After washing, mouse monoclonal anti-c-Myc (BioLegend, San Diego, USA) diluted 1:3,000 in PBST was added and kept at room temperature for 1 h. The horse-radish-peroxidase conjugated goat anti-mouse (GE Healthcare, Dako, Denmark, EU) (diluted 1:5000 in PBST) was added and kept for 1 h. After washing, ABTS (2,2'-Azinobis [3-ethylbenzothiazo-line-6-sulfonicacid-diammonium salt) chromogenic substrate (KPL. Inc, USA) was added. The absorbance OD at 405 nm was measured. The *E.coli* lysate clone which gave OD signals two times higher than control was selected for the next experiment.

Expression and purification

The selected XL-1 Blue *E.coli* transformed with *muscfv*-pOPE101 were grown in 100 mL LB-broth supplemented with 100 µg /mL ampicillin incubated at 37 °C with shaking. The protein expression was induced by Isopropyl-β-D-thiogalacto-pyranoside (IPTG) (Sigma-Aldrich, a final concentration of 1 mM. Cells were harvested 3 h later, centrifuged at 10,000 rpm for 20 min at 4 °C, and lysed by sonication in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8). The *E.coli* lysate was collected and subjected to Ni-NTA resin (Thermo Scientific, USA) affinity chromatography purification system according to the manufacturer›s instructions. The purified anti-AA *MuscFv* protein was aliquoted and stored at -20 °C until use. The purity of purified *Muscfv* was determined by SDS-PAGE and Coomassie staining.

DNA characterization and sequences

DNA sequences from selected anti-AA *MuscFv* clones were sequenced using pelB (5›-ATACCTATTG-CCTACGGCAGC-3›) and pOPE101 (5› -TAGATCTTCTTCTGAGATCAGC -3›) primers. The determining regions (CDRs) and immunoglobulin framework regions (FRs) of *muscfv* sequences were analyzed using (IMGT/V-QUEST tool of the International ImMunoGeneTics Information System (IMGT®) [18]. The DNA sequence of anti-AA *MuscFv* was multiple aligned using Bio-Edit and ClustalW2 program.

Homology modeling and intermolecular docking

Anti-AA *MuscFv* sequences were aligned using Bio-Edit and the ClustalW2 program. The position of VH, VL, and Linker according to the vector pOPE101 was determined for validation of protein structure and translated to amino acid via the website (https://-web.expasy.org/translat). Anti-AA *MuscFv* submitted the sequence of each protein to be modeled using the website (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/Alpha-Fold2.ipynb). The best match model of each protein (antigen and antibody (*scFv*)) was submitted for docking. The acrylamide 3D structure modeled

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pdb files for docking assembly were searched (https://www.rcsb.-org/). Docking results were obtained according to their binding affinities by using AutoDock Vina. PyMOL. Discovery Studio was used for the largest docking clusters of the interactive residues, and those with the lowest local energy were selected.

Determination of the cutoff value of Anti-AA *MuscFv* antibody by indirect ELISA

To establish the cutoff value of the indirect ELISA, 10-fold dilutions of the AA standard were used. Each dilution was tested in triplicate. The $OD_{405\ nm}$ value plus three times the standard deviation (SD) was used as the cutoff [19]. All experimental samples were considered positive if the $OD_{405\ nm}$ value was higher than this cutoff value.

Determination of Anti-AA *MuscFv* specific to acrylamide in coffee

The validity of Anti-AA *MuscFv* specific to acrylamide in coffee was conducted by using 2 brands of dark roasted coffee beans; designated coffee A and coffee B. The spike (0.1, 0.3, 0.5, and 0.7 mg/mL) and non-spike with AA standard in coffee was determined by ELISA as protocol previously described above. Furthermore, the presence of acrylamide in coffee was also confirmed by High-performance liquid chromatography (HPLC).

Results

Selection of anti-AA *MuscFv* antibody specific to acrylamide

After bio-panning, the *E.coli* colony containing recombinant phagemid *muscfv* vector was individually picked for direct colony PCR. Five clones (21.7%) presented positive PCR bands at expected size; ~750 - 900 bp as shown in Figure 1a. All positive clones were selected for protein expression. All 5 clones (100%) could be expressed *MuscFv* at molecular size ~25-30 kDa. The ELISA binding activity of these 5 clones is shown in Figure 1b. According to sequencing results and ELISA binding, clone number 11 was selected for sub-clone into the pOPE101 vector for large-scale recombinant protein expression and purification. Colony-directed PCR screening showed four positive PCR amplicons (N11.1, N11.2, N11.3, and N11.4) from 7 colonies screening (71.4%) Figure 1c Protein expression of all 4 clones showed the positive band at the expected molecular mass (~25 - 30 kDa) as shown in Figure 1d. All 4 clones could bind to acrylamide by ELISA binding result (data not shown).

Expression and purification of anti-AA *MuscFv* antibodies

After sub-cloning into the pOPE101 vector, direct colony PCR was performed. The positive PCR at ~750 - 900 bp was selected for protein expression. Clone number 11.1 (M*scFv*-N11.1) was

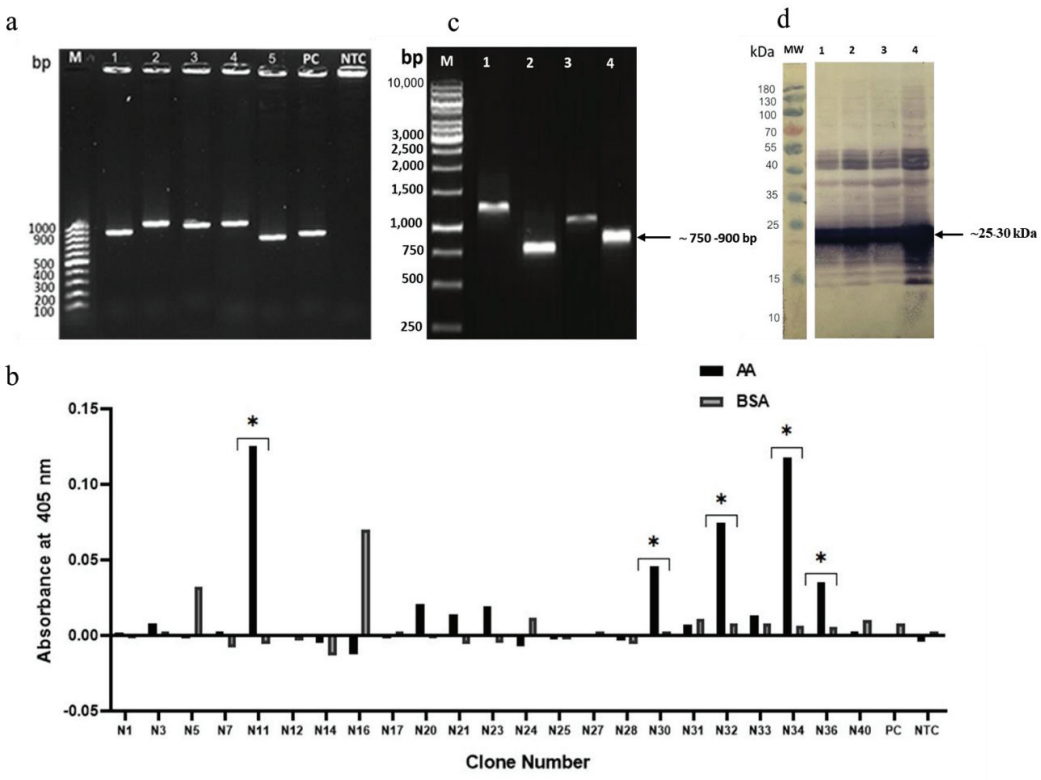

Figure 1: Selection of *MuscFv* antibody specific to acrylamide. **a)** Representative of *MuscFv* PCR amplicon at the expected size ~750 -900 bp. There are 21.7% positives selected from the muscfv phage library. b) ELISA binding of *MuscFv*, all 5 clones could bind to acrylamide. According to the sequencing result, clone number 11 was chosen for further transformation into a pOPE101 vector for large-scale protein expression. c PCR amplicon from directed colony PCR screening of *MuscFv* after transformation into pOPE101 vector, the result showed that from positive 4 clones from 7 colonies (71.4%). d Protein expression of all clones showed the positive band at the expected molecular mass (~25-30 kDa).

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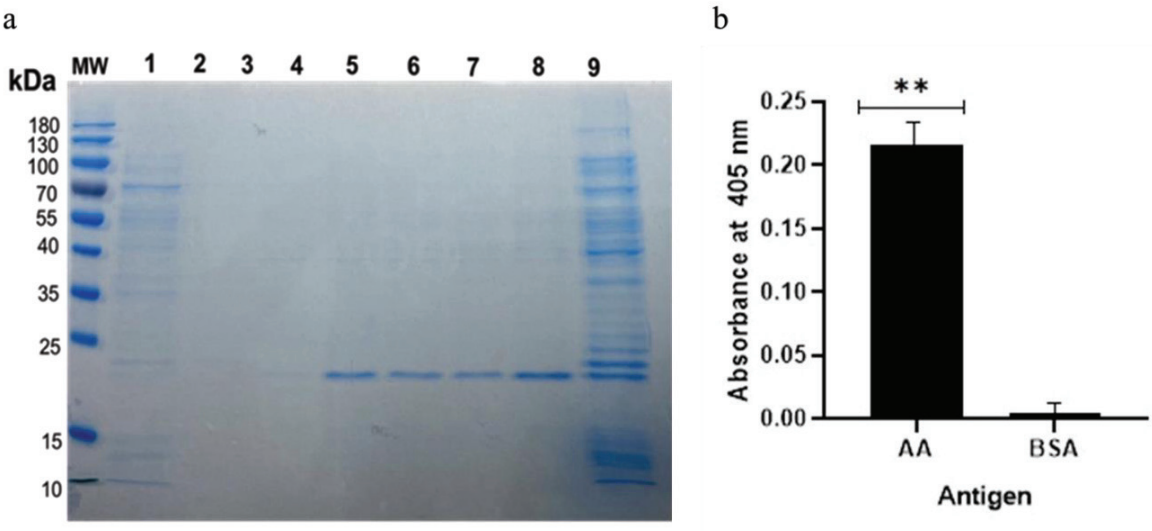

Figure 2: a) SDS-PAGE of purified *MuscFv*-N11.1 at the expected molecular mass (~25 - 30 kDa). The expected positive band was obtained. Lanes number 1 was unbound *E. coli* lysate; lane number 2 was washed fraction; lanes 3 - 8 were eluted fraction (E1-E5) and lane 9 was *E. coli* lysate. **b)** ELISA binding result of *MuscFv*-N11.1 showed statistically significant specific binding of *MuscFv* to acrylamide when compared with BSA ($p < 0.001$).

selected. Figure 2a showed Coomassie staining of the purified *MuscFv*-N11.1 protein.

Purified anti-AA *MuscFv* antibodies bound specifically to acrylamide

The binding activity of purified *MuscFv*-N11.1 was verified via indirect ELISA. Coated AA and BSA wells were incubated sequentially with *MuscFv*-N11.1, mouse monoclonal anti-c-My, HRP-conjugated goat anti-mouse IgG, and ABTS substrate, respectively. The absorbance OD at 405 nm was shown in Figure 2b. The result showed that *MuscFv*-N11.1 could bind specifically to AA ($p < 0.001$) when compared to BSA.

Characterization and homology modeling and intermolecular docking

The sequence analysis showed a complete CDR1-3 and immunoglobulin framework of mouse VH and VL as shown in Figure 3. This should be indicated that *MuscFv*-N11.1 was successfully constructed. The molecular docking model of *MuscFv*-N11.1 was performed. Ribbon display model showing *MuscFv*-N11.1 in blue, green, gray, and red. Superimposed picture of the 3D structures of *MuscFv*-N11.1 and acrylamide structures as shown in Figure 4a. *MuscFv*-N11.1 used D-58, D-60, and Y-105 of VH domain to interact with acrylamide via hydrogen bond (energy rang - 12.89 kcal/mol) as shown Figure 4b)

Validation of AA standard and anti-AA *MuscFv* antibody

The checker broad titration was used to determine the lowest amount of acrylamide detected (LOD) and the lowest amount of Anti-AA *MuscFv* antibody used in the ELISA. The AA standard was diluted in coating buffer; 0, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, and 1 mg/mL and coated in each ELISA well. A concentration of 0.5 mg/mL was found to be the lowest concentration that could be detected and gave similar OD405 as the concentration 0.6 and 0.8 mg/mL, respectively. Whereas, the lowest amount of Anti-AA *MuscFv* antibody was 0.8 mg/mL (Figure 5). Triplicate wells of each concentration were performed.

After the acrylamide standard›s test linearity plot. Figure 6a illustrates the linear correlation between concentration and area under the peak was found to be linear across the measured concentration range, with an $R^2 = 0.9303$. To establish the cutoff value of the ELISA, the AA standard was 10-folded diluted, and analyzed. The mean of the $OD_{405\ nm}$ values for these samples, as detected by the indirect ELISA, was 0.0510, with a standard deviation of 0.0003, which was calculated using the formula: mean of the negative sample values plus three standard deviations (SDs) [19] (Deshpande For a 99% confidence interval, the cutoff was defined as follows: mean of the negative serum $OD_{405\ nm}$ values plus three standard deviations = 0.0510 + 3 × 0.0003 = 0.0519. The sensitivity of this in-house ELISA was 0.005 mg/mL as shown in Figure 6b. Spike-and-recovery and linearity-of-dilution experiments are important methods for validating and assessing the accuracy of ELISA [20]. The concentration of the positive control sample (known concentration) was within the linear section of the standard curve in order to obtain valid and accurate results (data not shown).

Determination of AA *MuscFv* specific for acrylamide in coffee

The efficacy of *MuscFv*-N11.1 specific to acrylamide in coffee was performed by ELISA. Two bands of coffee; coffee A and coffee B were randomly selected. The *MuscFv*-N11.1 could detect acrylamide in both spiked and non-spiked coffee as shown in Figure 7a. The bar graph shows an increasing trend that varies with the concentration of AA standard (**). For coffee A and coffee B 0 mg/mL served as the negative controls (NC) (*) were able to detect acrylamide in coffee A and coffee B at similar

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MAQVTLKESGPGILQPSQTLSLTCSFSGFSLNTSGMGVSWIRQPSGKGLEWLAHIYWDDDKRYNPSLKSRLTI 73
VH-CDR1 VH-CDR2
SKDTSRNQLFLKITSVDTADTATYYCARTGDYDYDDRFAYWGQGTLVTVSAAKTTPPKLEEGEFSEARVDIVIT 147
VH-CDR3 Linker
QDELSNPVTSGESVSISCRSSKSLLYKDGKTYLNWFLQRPGQSPQLLIYLMSTRASGVSDRFSGSGSGTDFTLEIS 223
VL-CDR1 VL-CDR2
RVKAEDVGVYYCQQLVEYPRTFGGGTKLEIK 254
VL-CDR3

Figure 3: The deduced amino acid (aa) sequence of *MuscFv*-N11.1 was shown including the VH and VL domains joined by a (Glu4 Gly1 Phe1 Ser1) linker, the complementary determining region 1-3 (CDR1-3).

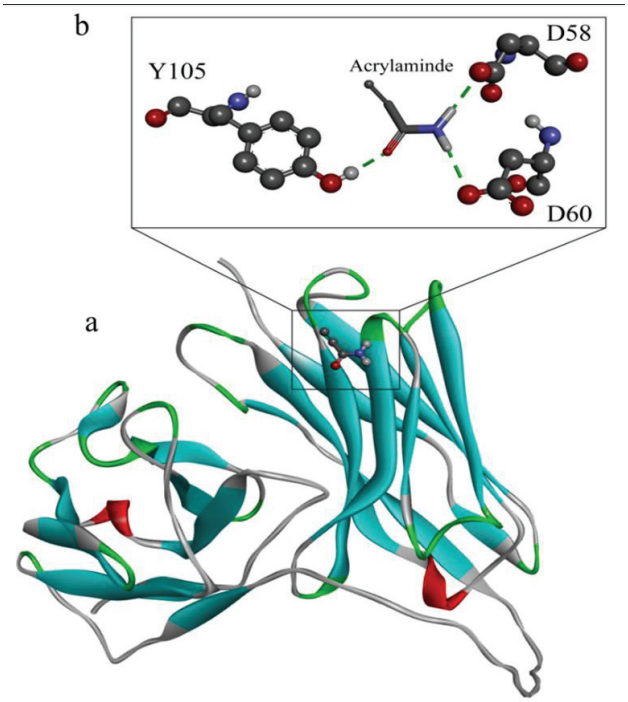

Figure 4: Molecular docking model of *MuscFv*-N11.1 interacts with acrylamide. **a)** Ribbon display model showed *MuscFv*-N11.1. **b)** Close-up view of *MuscFv*-N11.1 and acrylamide used D-58, D-60, and Y-105 of VH domain to interact with acrylamide via hydrogen bond.

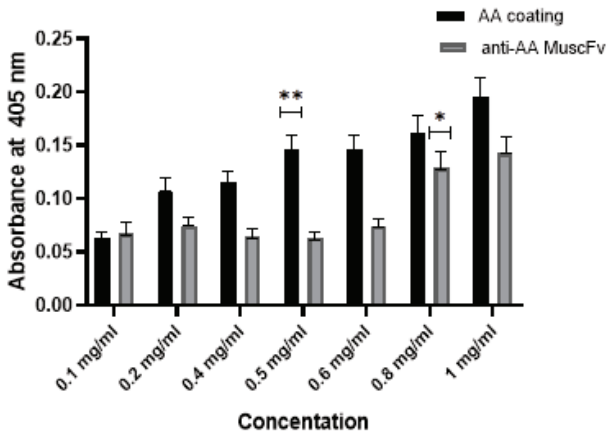

Figure 5: Checker broad titration results showed the lowest amount of acrylamide (LOD) was 0.5 mg/ml (black bar) (**) and gave similar OD405 concentrations of 0.6 and 0.8 mg/ml, respectively. The lowest amount of anti-AA *MuscFv* antibody that could be used in ELISA was 0.8 mg/ml (grey bar) (*). Each experiment was performed in triplicate. Values were presented as the means ± SD.

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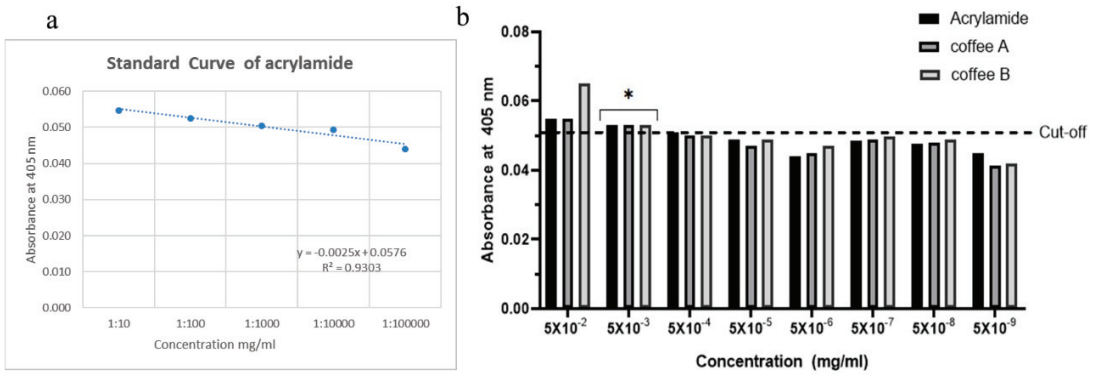

Figure 6: a) The acrylamide standard test linearity plot correlation between concentration and area, with an R2 = 0.9303. **b)** The cutoff value of *MuscFv*-N11.1 specific to acrylamide was performed by the ELISA, the result cutoff value = 0.0519. The sensitivity of this in-house ELISA was 0.005 mg/mL ($5X10^{-3}$ mg/mL).

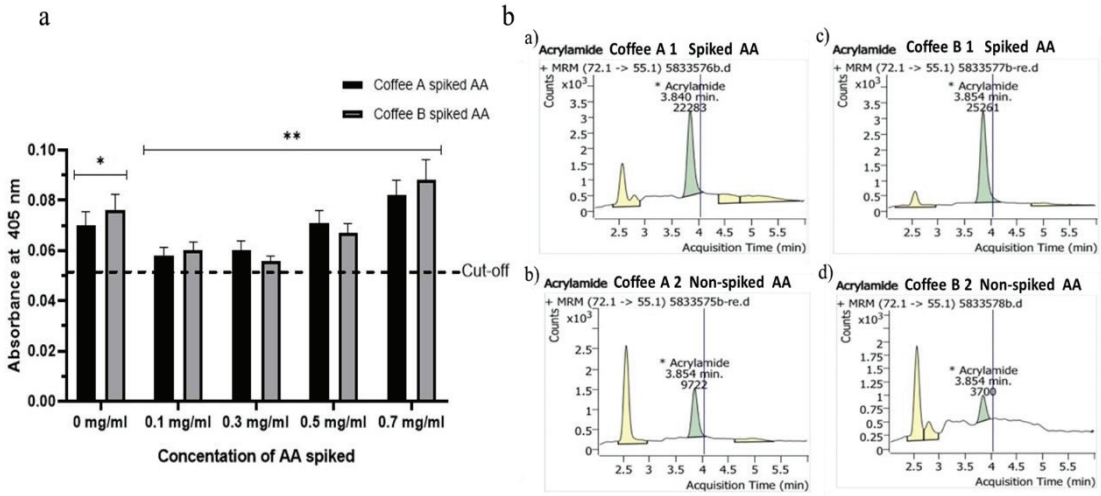

Figure 7: a) The determination of *MuscFv*-N11.1 specific to acrylamide in coffee was performed using the dark roasted coffee beans containing 2 coffee brands, coffee A (black) and coffee B (grey) spiked with AA standard at concentrations of 0, 0.1, 0.3, 0.5, and 0.7 mg/mL. Furthermore, the non-spike AA also showed positive results for acrylamide presentation. **b)** Chromatograms of acrylamide standard 1000 ng/mL, AA-spiked coffee A and B and non-AA-spiked; All could detect acrylamide in these 2 brands of coffee (Figure 7B.A-7B.D), respectively.

concentrations (0.5 and 0.7 mg/mL). Each experiment was performed in triplicate. Values were presented as the means ± SD. All experimental samples› values were higher than the cutoff value (0.0519). The result was also confirmed by HPLC that both bands of coffee have acrylamide in Figure 7b. The spiked AA in coffee showed an OD_{405} increase when the AA spiked concentration increased, which was shown in Figure 7a. The result revealed a dose-dependent fashion. Thus, the produced *MuscFv*-N11.1 could bind specifically to acrylamide.

Discussion

Acrylamide belongs to a group of toxins that is harmful to human and animal health, due to both acute and chronic effects, including neurotoxicity, genotoxicity, carcinogenicity, reproductive toxicity, hepatotoxicity, and immunotoxicity [21]. The presence of acrylamide was found predominantly in heat-treated carbohydrate-rich foods, such as potatoes, biscuits, cereals, and coffee; announced by the Swedish National Food Administration in April 2002 [22]. The European Food Safety Authority (EFSA) has determined an estimated dosage range within which the substance is likely to cause potential health effects. Acrylamide has been estimated to range from 425 for average adult consumers down to 50 for high consuming toddlers. In coffee determined it to 400 µg /kg for roasted coffee and not more than 850 µg /kg for instant coffee [23]. Among a variety of established methodologies for analyzing acrylamide, namely high-performance liquid chromatography (HPLC) [8], gas chromatography coupled with mass spectrometry (GC-MS) [9,10] and immunoassay [13] etc. were described. The immunological methods are among the most rapid, simplest, cheapest, and most suitable for on-lab screening.

ELISA, a rapid method based on the recognition of antigen-antibody binding with high specificity and affinity, utilizes optical detection of colored products catalyzed by enzyme labels. Due to its specificity and affinity, coupled with efficient enzymatic catalysis, ELISA methods offer optimal recovery

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and adaptability for detecting AA in various food samples, including Chocolate products, Cocoa powder, Nuts, French fries, roast potatoes, potato crisps/chips, biscuits, baked, and toasted [21]. The advantages such as affordability, simplicity, ease of handling, and portability, prove particularly effective in identifying AA in thermally processed foods [24]. ELISA also demonstrates good sensitivity, selectivity, high-throughput capabilities, and compatibility with other technologies like biotin-avidin amplification and chemiluminescence, thus attracting increased attention for AA detection in foods [25]. However, the multiple washing and incubation steps in ELISA extend the detection process, making it more suitable for primary screening of food products with excessively high AA concentrations during processing or on the market. In comparison, standard methods like LC-MS/MS and GC-MS provide rapid detection, meeting the needs of food industries, regulatory bodies, and consumers [26]. Nevertheless, they require further refinement to enhance accuracy, sensitivity, repeatability, reproducibility, multi-step, and portability for achieving online and real-time detection of trace amounts of AA [27].

In this study, mouse variable heavy and light chain formed of *MuscFv*-N11.1 was selected from the *muscfv*-phage library. The *muscfv* -phage library size utilized in this experiment with substantial antibody sequence diversity, which was comparable to the other non-immune libraries previously reported [28,29]. Similarly, in a related study using an immunized phage display library a specific nanobody termed Nb-7E against an acrylamide derivative xanthyl acrylamide (XAA) was isolated from an immunized phage display library and confirmed to be able to detect acrylamide [25]. *MuscFv*-N11.1 was found to be specific to acrylamide by using established ELISA. The result confirmed by HPLC that both bands of coffee have acrylamide. The chromatograms showed acrylamide peak and interference corresponding to an acrylamide calibration standard with a retention time of 3.8 min. Similarly, a study in-depth study of acrylamide formation in coffee during roasting: role of sucrose decomposition and lipid oxidation using determination of acrylamide in coffee by liquid chromatography-tandem mass spectrometry. Acrylamide peak showed a retention time of 2-3.1 min [30]. Corresponding to the study determination of acrylamide after Its extraction from potato chips was acrylamide spiked in chips sample and analyzed using ultrasound-assisted liquid-liquid extraction (UA-LLE) technique / HPLC-UV showed acrylamide peak at a retention time of 4.15 min [31]. Computerized docking models showed that *MuscFv*-N11.1 used the VH domain (D-58, D-60, and Y-105) to interact with acrylamide via hydrogen bonds. The binding efficacy of *MuscFv*-N11.1 to acrylamide was less than 0.005 mg/mL.

Conclusion

In this study, the selection of antibodies against acrylamide from murine *scFv* phage-displayed library was performed in order to select used by acrylamide standard: AA and N-acryloxy succinimide: NAS used as antigen for bio-panning were expressed and purified to be specific for acrylamide contaminated in food. Anti-AA *MuscFv* against acrylamide was produced, and an indirect ELISA was developed for the determination of coffee. The application to samples from coffee indicated that ELISA may be used for the estimation of total acrylamide concentrations. The method has shown satisfactory results in terms of specificity and accuracy, which were confirmed by High-Performance Liquid Chromatography (HPLC).

Acknowledgement

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Ethics statement

This study protocol was reviewed and approved by the Biosafety Control Board at Suranaree University of Technology, Nakhon Ratchasima, Thailand (SUT-IBC-03/2021).

Author contributions

SP performed most experiments and drafted the manuscript. JT carried out the selection of the phage library against acrylamide and contributed to the genetic, molecular, and immune biochemical characterization of *MuscFv*. In addition, SP participated in the design and coordination of the study. PS and NN performed some of the experiments such as purification and bio-panning procedures for *muscfv* isolation and selection. TN performed some of the experiments such as designing ELISA for binding testing. SS, TS, and KTI helped to consult in the lab and draft the manuscript. Furthermore, KTI participated in the design and coordination of the entire research project. All authors have read and approved the final version of the manuscript.

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