

Anisakis haemoglobin is a main antigen inducing strong and prolonged immunoreactions in rats

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Abstract *Anisakis simplex* larvae are well known to cause gastrointestinal and allergic manifestations after ingestion of parasitized raw or undercooked seafood. The antibody recognition dynamics against the components of *Anisakis* larval antigen after primary and re-infection with *Anisakis* live larvae remain unclear. For this study, immunoblot analyses of serum IgG, IgE, and IgM against *Anisakis* larval somatic extract were performed in rats that had been orally inoculated with *A. simplex* live larvae. Multiple antigen fractions were recognized after primary infection. Their reaction was enhanced after re-infection. Antibody recognition was observed for 12 weeks after re-infection. The fraction of approximately 35 kDa contained a main antigen that induced strong and prolonged immunoreactions in IgG and IgE. The antibody reaction to this fraction appeared to be enhanced after inoculation of larval homogenates. This fraction was heat tolerant with boiling for 30 min. The fraction was spotted by immunoblotting after two-dimensional electrophoresis and was identified as *Anisakis* haemoglobin (*Ani s 13*) using mass spectrometry analysis. The amino acid sequences of haemoglobin mRNAs from two *A. simplex* sensu stricto and one *Anisakis pegreffii* were identified by RACE-PCR. They

differed from those of two isolates of *Pseudoterranova decipiens* and *A. pegreffii*. Results of this study show that *Anisakis* haemoglobin, which is known to be a major allergen of *A. simplex*, induces strong and prolonged immunoreaction in rats. This report is the first to show the amino acid sequence variation of *Anisakis* haemoglobin mRNA between *A. simplex* sensu stricto and *A. pegreffii*.

Keywords *Anisakis simplex* · *Anisakis* haemoglobin · Immunoblotting · Two-dimensional electrophoresis · Mass spectrometry · Rat

Introduction

Anisakis simplex larvae are well known to cause gastrointestinal and allergic manifestations after ingestion of parasitized raw or undercooked seafood (Nieuwenhuizen and Lopata 2013). To date, many immunological studies of this nematode have been conducted using sera from patients or experimental animals infected with this nematode, but antibody recognition against *Anisakis* larval antigen has been demonstrated only in several studies using experimental animals (Iglesias et al. 1993; Amano et al. 1995; Perteguer and Cuéllar 1998; Baeza et al. 2005; Cho et al. 2006; Abe and Teramoto 2014). Nevertheless, these studies do not fully elucidate antibody recognition against the larval antigen components after primary infection and re-infection with live larvae because their evaluation was performed solely by enzyme-linked immunosorbent assay (ELISA) (Amano et al. 1995; Perteguer and Cuéllar 1998; Abe and Teramoto 2014) or ELISA with immunoblotting using a few serum samples collected during the experimental period (Iglesias et al. 1993; Baeza et al. 2005; Cho et al. 2006). Currently, 14 *Anisakis* antigens have been identified as major or pan-allergens (Nieuwenhuizen and

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Lopata 2014; González-Fernández et al. 2015; Kobayashi et al. 2015). It is necessary to elucidate the dynamics of antibody recognition against the *Anisakis* larval antigen components, especially these allergens, after primary infection and re-infection for analysing the immunogenicity of *Anisakis* antigens. For the present study, we elucidated the dynamics of antibody recognition against *Anisakis* larval antigen by immunoblotting using sera from rats that were inoculated twice with live *Anisakis* larvae. Results show that the 13th *Anisakis* allergen, *Ani s 13*, is a major antigen that induces strong and prolonged immunoreaction after primary infection and re-infection with live *Anisakis* larvae. Moreover, this report is the first study demonstrating the amino acid sequence variation of *Anisakis* haemoglobin between *A. simplex* sensu stricto and *Anisakis pegreffii*.

Materials and methods

Oral inoculation of live larvae to rats

The third-stage larvae of *A. simplex* (L3) were collected from viscera of chub mackerel (*Scomber japonicus*). As shown in Fig. 1, the six 8-week-old male Wistar rats (Japan SLC, Inc., Japan) were inoculated and re-inoculated orally with 15–20 live L3 and then they were inoculated orally with larval homogenates from 15 L3 after re-inoculation. L3 homogenates were prepared as mentioned below. All rats were bled from the tail once every week until 2 weeks after inoculation of larval homogenates. Sera from normal rats before inoculation of live L3 were used as negative control. Pool sera from six rats at each bleeding point were used for the following immunoblotting assay. Successful *Anisakis* infection in all animals was confirmed using ELISA (Abe and Teramoto 2014) (data not shown).

Somatic extract and immunoblotting

The L3 collected from viscera of chub mackerel were washed with phosphate-buffered saline (PBS) to remove fish contaminants and were then frozen at -80°C for 120 min. They were suspended in PBS containing protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific Inc.,

USA). After they were ruptured using microtube pestles (Scientific Specialties, Inc., USA), they were sonicated on ice and centrifuged at $105,000\times g$ for 60 min at 4°C . The larval homogenates inoculated to animals mentioned above were prepared without centrifugation. Protein concentrations of somatic extract were estimated using a kit (Quick Start Bradford Protein Assay; Bio-Rad Laboratories Inc., USA) and were stored at -20°C until use.

Worm somatic extracts were dissolved in $4\times$ Laemmli sample buffer with 2-mercaptoethanol and boiled for 5 min as a reducing condition. SDS-PAGE was performed using a Mini Protean Tetra cell and Mini Protean TGX gradient (4–20%) IPG gels (Bio-Rad Laboratories). The protein bands were transferred onto a $0.2\text{-}\mu\text{m}$ pore size PVDF membrane using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories) and blocked with Blocking One (Nacalai Tesque, Inc., Japan). After washing the membrane with tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), it was put in the Screener Blotter with 12 channels (Sanplatec Corp., Japan). The sera from *A. simplex*-infected rats diluted at 1/100 with 5% Blocking One in TBST (BO-TBST) were put into each channel and incubated at 4°C overnight. The membrane was removed from a Screener Blotter after washing each channel with TBST. Horseradish peroxidase (HRP)-labelled goat anti-rat IgG (H&L) and IgM (H&M) (American Qualex Scientific Products, CA, USA) diluted at 1/2000 with BO-TBST were used as secondary antibodies for the detection of *Anisakis*-specific IgG and IgM, respectively. For the detection of *Anisakis*-specific IgE, mouse monoclonal anti-rat IgE (MARE-1; American Research Products, Inc., USA) diluted at 1/200 with BO-TBST and HRP-labelled goat anti-mouse IgG (H&L) (American Qualex) diluted at 1/1000 with BO-TBST were used as the secondary and third antibodies, respectively. To visualize bands, the membrane was treated with a mixed solution of peroxidase stain DAB and metal enhancer for DAB stain (Nacalai Tesque). To test heat resistance, a safe-lock tube including somatic extract was incubated for 30 min in boiling water. The other two tubes including somatic extract were autoclaved at 90°C for 115 min or 121°C for 20 min. The tubes were centrifuged and the supernatants were used for immunoblotting with IgG and IgE using pool sera at 2 weeks after inoculation of larval homogenate.

Two-dimensional electrophoresis (2D) and 2D-immunoblotting

The larval somatic extract was treated with EzApply 2D kit (Atto Corp., Japan) for first-dimension electrophoresis (IEF). The treated antigen containing approximately $200\text{ }\mu\text{g}$ of total protein was used for each precast agarose disc gel (pH 5–10) (agarGEL; Atto). IEF was performed using a Mini Disc gel electrophoresis system (WSE-1500 Disc Gel EP kit; Atto). After focusing, the gels were soaked in 2.5% trichloroacetic

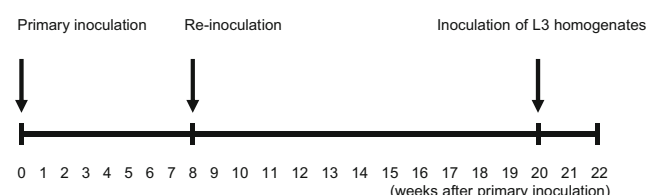


Fig. 1 Schematic diagram of inoculation and re-inoculation of L3. All animals were re-inoculated with the same number of live L3 (15–20 L3/rat) at 8 weeks after primary inoculation and were then inoculated with larval homogenates from 15 L3 at 12 weeks after re-inoculation

acid solution and were then washed using distilled water. After washing, the gels were soaked in equilibration solution (50 mM Tris-HCl pH 6.8, 2% SDS, 0.01% bromophenol blue). Second-dimension gel electrophoresis was performed using a Mini-slab size electrophoresis system (Atto). The equilibrated strip gel was placed onto e-PAGE mini gel (gradient 5–20%, IPG; Atto) to perform the SDS-PAGE. In 2D analysis, two electrophoresis gels were run in pairs. One was used for confirming the spots around approximately 35 kDa after development using the kit (Rapid Stain CBB; Nacalai Tesque). The other gel was transferred onto a 0.2- μ m pore size PVDF membrane as mentioned above. The membrane was developed using a kit (Rapid Stain CBB) to confirm the same-sized spots. After confirmation of the spots, the membrane was destained using the Rapid CBB Destain Kit (Nacalai Tesque) and was then used for immunoblotting with serum IgG and IgE.

Protein identification by mass spectrometry analysis

Approximately 35 kDa spot confirmed by 2D-PAGE and immunoblotting was cut from the other stained gel processed similarly. It was provided for Nano LC-MS/MS analysis and Mascot Search by JBioS (Japan Bio Services Co. Ltd., Japan). The following search parameters were used: type of search, MS/MS Ion Search; enzyme, trypsin; variable modification, carbamidomethyl (c), oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ± 0.2 Da; fragment mass tolerance, ± 0.2 Da; max missed cleavages, 1; instrument type, default. The confidence interval for protein identification was set to $\geq 95\%$ ($p < 0.05$). Peptides with individual ion scores higher than the identity threshold were regarded as correctly identified.

Identification of amino acid sequences of *Anisakis* haemoglobin mRNA from *A. simplex* sensu stricto and *A. pegreffii*

The 10 *Anisakis* larval nematodes from the viscera of a chub mackerel were provided for the extraction of DNA and RNA. The DNAs were extracted from very small amounts of the body cut from a tail end using a QIAamp DNA mini kit (Qiagen GmbH, Germany) and all nematodes were identified at the sibling species level by *Anisakis simplex*-specific PCR and PCR-RFLP of SSU rDNA (Zhu et al. 1998; Szostakowska et al. 2002). The other parts of the body were provided for the extraction of total RNA using a kit (NucleoSpin RNA; Macherey-Nagel GmbH and Co. KG, Germany), and the three RNA samples from two *A. simplex* sensu stricto and one *Anisakis pegreffii* were provided for the rapid amplification of the haemoglobin cDNA ends (RACE) using the SMARTer RACE 5'/3' kit (Clontech Laboratories, Inc., USA) with the following newly developed primer pair:

#21 (5'-GGA ACC ATT ATG CAC TCT TC-3') and #23 (5'-TTC AGT GGT GTT CCT CCT TG-3') under the reaction condition of this kit (www.clontech.com/manuals). The resulting PCR products were cloned (Target Clone; Toyobo Co. Ltd., Japan). The positive clones were selected using the Insert Check Ready (Toyobo) and were sequenced in both directions on an automated sequencer (ABI 3130; Thermo Fisher Scientific). The sequences were translated amino acid sequences and were aligned with the two previously deposited haemoglobin sequences from *Pseudoterranova decipiens* and *A. pegreffii* using the MUSCLE program in MEGA 6 (Tamura et al. 2013). The nucleotide sequences of *A. simplex* sensu stricto and *A. pegreffii* mRNA for haemoglobin reported herein were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC209224 (isolate #13, *A. pegreffii*), LC209225 (isolate #2, *A. simplex* sensu stricto) and LC209226 (isolate #11, *A. simplex* sensu stricto).

Results and discussion

As shown in Fig. S1, immunoblotting showed multiple components in L3 somatic extract, which were recognized by IgG (Fig. S1A), IgE (Fig. S1B), and IgM (Fig. S1C). Especially, approximately 14 kDa (indicated by arrowhead) and 35 kDa (arrow) fractions were apparent in IgG and IgE. In immunoblotting with IgE, an approximately 35 kDa fraction was recognized at 2 weeks after primary inoculation (lane 2 in Fig. S1B) and its reactivity (reactivity is indicated by the colour intensity judged not quantitatively but visually) was markedly weaker at 6 and 8 weeks after inoculation. However, the reactivity to this fraction was enhanced at 1 week after re-inoculation with live L3 (lane 5 in Fig. S1B). It appeared to be no different among 8–12 weeks after re-inoculation (lanes 7–10 in Fig. S1B). It is particularly interesting that the reactivity to this fraction was apparently enhanced at 1–2 weeks after inoculation with larval homogenates (lanes 11 and 12 in Fig. S1B). Like that from immunoblotting with IgE, the reactivity for this fraction by IgG appeared to be enhanced at 1–2 weeks after inoculation with larval homogenates (lane 11 and 12 in Fig. S1A). The positive reaction in IgG and IgE for an approximately 14 kDa fraction was found at 6 weeks after primary inoculation. It was enhanced at 4 weeks after re-inoculation with live L3 (lane 6, Figs. S1A and S1B). Like that in an approximately 35 kDa fraction, reactivity to an approximately 14 kDa also appeared to be enhanced at 1–2 weeks after inoculation with larval homogenates (lane 11, 12, Figs. S1A and S1B). However, the immunoblotting pattern with IgM differed from those of IgG and IgE (Fig. S1C). The reactivity with IgM to an approximately 130 kDa smeary fraction (dot arrow) was readily observable after primary inoculation. It was enhanced at 1 week after re-inoculation with live L3, as compared with that in IgG. It is particularly

interesting that the reactivity to this fraction appeared to be enhanced at one week after inoculation with larval homogenates (lane 11, Fig. S1C). The reactivity to an approximately 35 kDa fraction was readily observable at 2 weeks after primary inoculation but it disappeared for 8 weeks after primary inoculation. After re-inoculation with live L3, the reactivity to this fraction was enhanced, but it weakened up to 12 weeks after re-inoculation (lanes 6–10, Fig. S1C). Unlike those in IgG and IgE, the positive reaction to approximately 14 kDa fraction was invisible during the experimental period. Immunoblotting with IgG, IgE, and IgM to somatic extract was performed an additional two times at different dates for confirmation of reproducibility of results, and the blotting pattern in each antibody isotype showed similar results among experiments.

Immunoblotting with IgG and IgE for heat-treated somatic extract showed that approximately 35 kDa fraction was heat tolerant for 30 min in boiling water (lane 1 in Fig. S2), but heat susceptible for 90 min at 115 °C (lane 2 in Fig. S2) or for 20 min at 121 °C (lane 3 in Fig. S2). In Japan, heat treatment with 90 min at 115 °C is applied for the sterilization of marine canned food. However, an approximately 14 kDa fraction was heat tolerant even at 121 °C for 20 min or 115 °C for 90 min, although the immunoblot reactivity was weaker than that processed for 30 min in boiling water. The reactivity with IgE to each heat-treated somatic extract was the same as that to IgG (data not shown).

As shown in Fig. S3A, the approximately 35 kDa fraction was divided in at least two spots (indicated by an arrow and dashed arrow) by 2D-page analysis. After immunoblotting with pool sera IgG, a strong positive reaction was found in only one fraction (Fig. S3B, arrow), but a faint positive reaction was found in the other fraction (Fig. S3B, dashed arrow). Reproducibility of the 2D-page and its immunoblotting patterns was confirmed by two re-experiments. The reactivity to both spots with IgE was the same with that of IgG (data not shown). Therefore, we identified that approximately 35 kDa fraction recognized by serum IgG and IgE from rats infected with live L3 was the same fraction.

Mascot Search using the data obtained from Nano LC-MS/MS analysis of an approximately 35 kDa spot shown by an arrow in Fig. S3 depicts this matched spot (score 313, matched peptide 16) with only haemoglobin from *A. pegreffii* (accession AFY98826), indicating the approximately 35 kDa spot recognized immunologically with IgG and IgE in rats is *Anisakis* haemoglobin.

In the present study, the 1009 bp sequences of mRNA for haemoglobin from two *A. simplex* sensu stricto and one *A. pegreffii* were clearly identified including the 5' end. The sequencing of haemoglobin mRNA from those isolates showed that they are most similar (97.1–97.5% identities within 931 bp overlap) to that of haemoglobin of *A. pegreffii* (JX860676) previously analysed (Nieuwenhuizen et al. 2013).

As shown in Fig. S4, the amino acid sequence in the hydrophobic leader portion was clearly identified in the present *Anisakis* sibling species, although it had not been obtained in the *A. pegreffii* isolate in the previous study (Nieuwenhuizen et al. 2013). The B10 tyrosine (Y), E7 distal glutamine (Q), F8 proximal histidine (H), important in binding of oxygen in two similar domains (Goldberg 1999), were conserved in *A. simplex* sensu stricto and *A. pegreffii* distributed in the Sea of Japan (Fig. S4). In addition, like *A. pegreffii* and *P. decipiens*, complete sequencing at the 3' end demonstrated *A. simplex* sensu stricto has one HKEE repeat. Previous study predicted epitopes of *Anisakis* haemoglobin based on its mRNA sequences of *A. pegreffii* (González-Fernández et al. 2015). In the present study, no amino acid differences were found among *A. simplex* sibling species in the epitopes 2 and 5, which is the most important epitopes in *Anisakis* haemoglobin for IgE binding and specificity. However, following amino acid substitutions were found among *Anisakis* sibling species: epitope 1, “L” at the position 56 was substituted “F” in the present three isolates; epitope 3, “HH” at the positions 308–309 were substituted “QY” in the present isolate 2 (*A. simplex* sensu stricto); epitope 4, “L” at the position 187 was substituted “A” in the present isolate 11 (*A. simplex* sensu stricto) (Fig. S4). In *Anisakiinae* nematode, the amino acid sequence of haemoglobin has been identified in only two species, *P. decipiens* (AAA29797, 333 aa) and *A. pegreffii* (AFY98826, 309 aa) (Dixon et al. 1991; Nieuwenhuizen et al. 2013), and it has been quite unclear in the other pathogenic *Anisakis* species, *A. simplex* sensu stricto, which is known as the main causative agent in anisakiasis in Japan (Umehara et al. 2007). Therefore, the present study demonstrated for the first time the amino acid sequence variation of *Anisakis* haemoglobin mRNA among *Anisakis simplex* sensu stricto and *A. pegreffii*.

In this study, multiple components of *Anisakis* larval antigen are recognized by serum IgG, IgE, and IgM from rats orally inoculated with *Anisakis* live larvae. Especially strong and prolonged reaction after primary inoculation and re-inoculation with live larvae were found in *Anisakis* haemoglobin (approx. 35 kDa) and 14 kDa fractions in IgG and IgE and approximately 130 kDa in IgM. The reactivity to *Anisakis* haemoglobin was readily apparent at 2 weeks and 1 week respectively after primary inoculation and re-inoculation with live larvae, but the reactivity was weakened or invisible thereafter. Therefore, the positive reaction to *Anisakis* haemoglobin by immunoblotting with IgM might reflect acute infection with *A. simplex* larvae in rats. González-Fernández et al. (2015) showed that recognition of *Anisakis* haemoglobin by IgG4 and IgE was higher in gastroallergic anisakiasis patients than in patients with chronic urticaria by antigen-capture ELISA. Therefore, measurement of *Anisakis* haemoglobin-specific IgM by ELISA or immunoblotting might be useful diagnostic tool for acute *Anisakis*

infection and gastroallergic anisakiasis in patients, although it is problematic to easily extrapolate the results of animal experiments to clinical practice.

An approximately 130 kDa fraction was not identified using mass spectrometry analysis because of its smeary fraction. However, Nano LC-MS/MS analysis showed two proteins in an approximately 14 kDa fraction: “myophilin (accession, ERG84874)” (15 kDa), muscle-specific antigen of *Ascaris suum*, which is classified in Ascarididae nematode as it is with *A. simplex*, and *Ani s 9* (ABV55106), which is known to be heat stable (boiling for 30 min) *Anisakis* ninth allergen (14 kDa) (Rodríguez-Pérez et al., 2008), suggesting that this fraction comprises at least two proteins. The *Anisakis* haemoglobin was recently identified as a major allergen (*Ani s 13*) recognized in numerous (64.3%) sensitized patients with cutaneous features after *Anisakis* parasitism and in up to 80.9% of patients with gastroallergic anisakiasis (González-Fernández et al. 2015). In the present study, results showed that this allergen induces strong and prolonged immunoreaction with IgG and IgE in rats orally inoculated with live *Anisakis* larvae. Therefore, a rat model of intragastric infection with live *Anisakis* larvae might be a useful model for analysing *Anisakis* haemoglobin immunologically.

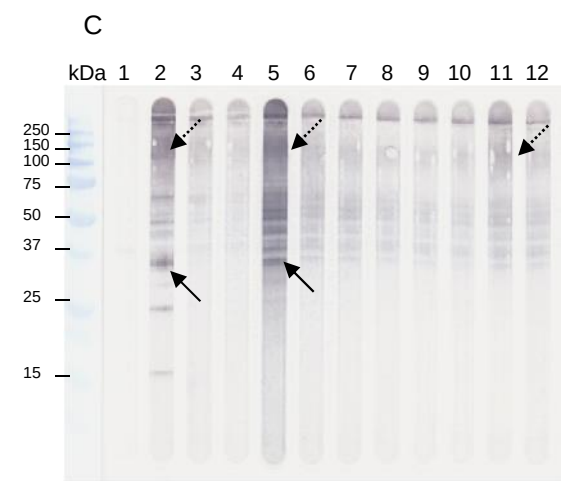
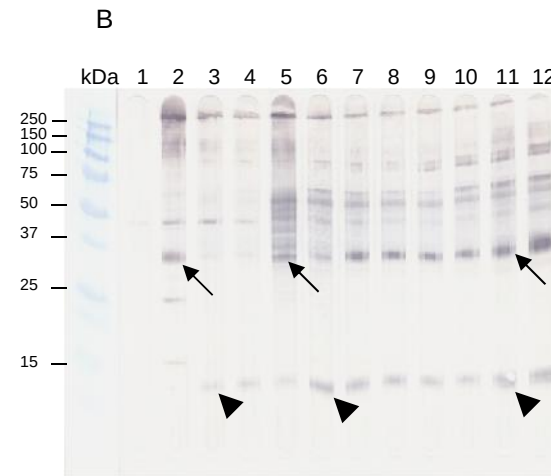
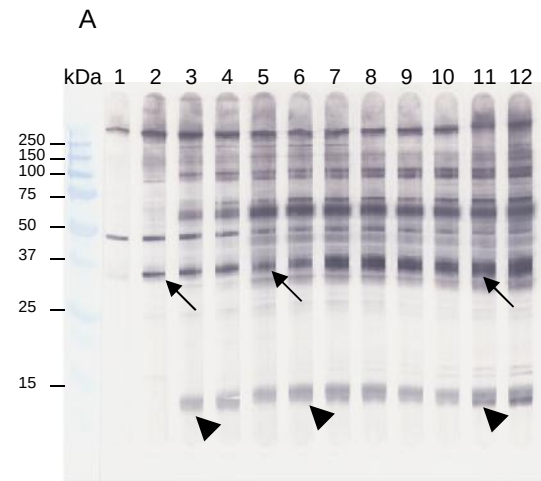
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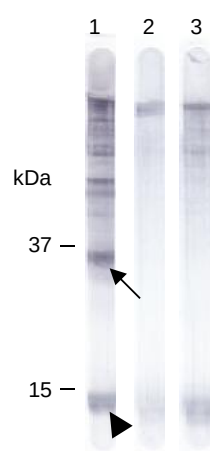
Compliance with ethical standards All animals were kept in an animal room of the Osaka City Institute of Public Health and Environmental Sciences according to the guidelines of the Experimental Animal Committee of this institution for laboratory animals.

Conflict of interest The authors declare that they have no conflict of interest.

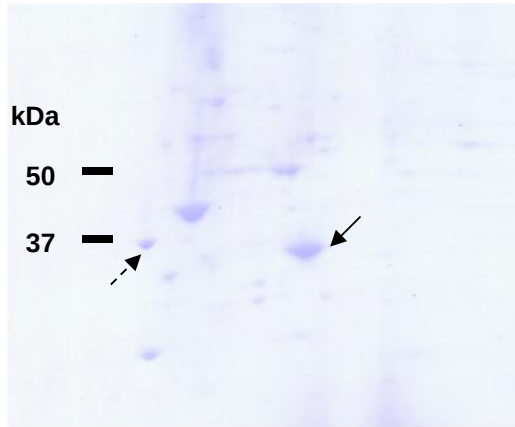
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B

