

Boronic acid-functionalized agarose affinity chromatography for isolation of tropomyosin in fishes

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Abstract

BACKGROUND: Tropomyosin is now receiving increasing attention because of its significant allergenic activity in various fishery products but its simple and effective isolation still remains a challenging task.

RESULTS: An agarose-based boronate affinity chromatography was produced for the first time to isolate tropomyosin in various fishery products using 3,5-difluoro-4-formyl-phenylboronic acid as the functional monomer, tris(2-aminoethyl)amine as the multi-branched ligand, and agarose gel particles as supporting materials. The agarose concentration, binding pH, and the concentration of elution buffers demonstrated significant effects on separation performance. Under optimized conditions, the purity of the isolated tropomyosin was higher than 90%, with the column adsorption capacity over 1.85 mg mL⁻¹ and the enrichment efficiency over 65%. Such efficiency was also validated with different fish samples including *Paralichthys olivaceus*, *Thunnus thynnus*, *Oreochromis spp.*, and *Lophius litulon*.

CONCLUSION: In comparison with conventional methods, the established affinity chromatography demonstrated excellent biocompatibility (without involving any organic solvent), better speed (from at least 1–2 days to 3–4 h), and simplicity (from at least five steps to three steps). This suggests that it is a novel and promising technique for the isolation of tropomyosin and other glycoproteins (including most allergens) in foodstuffs.

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Keywords: boronate affinity chromatography; glycoproteins; Tropomyosin; isolation

INTRODUCTION

Food allergy is now a major public health concern. Tropomyosin is an important allergen in many invertebrates such as shrimp, crab, and shellfish,¹ and this has been identified in the WHO/IUIS Allergen Nomenclature Database.² In recent years, tropomyosin in some fishes (for example *Oreochromis spp.*, *Lophius litulon*, *Thunnus thynnus*, *Thunnus alalunga*, and *Barchidano rerio* var.) was also reported to display significant allergenic activity and therefore attracted increasing attention as a result of the worldwide consumption of these aquatic products.^{3–5} An understanding of the mechanism involved in the tropomyosin-induced allergy in fishes and other aquatic products, as well as reasonable monitoring and labeling of tropomyosin in different food stuffs, now seems very important for the effective control of this hazard.

Preparation of high-purity tropomyosin and other allergens is the key for characterizing its physical, chemical, and biological properties in various fishery products. It is also necessary for the establishment of analytical techniques such as various immunoassays and mass spectrum (MS)-based methods. Conventional approaches are mainly performed as follows:^{6–8} Extraction with organic reagents to obtain crude proteins, followed by isoelectric point precipitation or ammonium sulfate precipitation for further separation, and then purification with chromatographic techniques, such as an ion exchange column, or sephadex G-50 chromatography. This kind of technique could result in relatively

large amounts of tropomyosin, but it is complex and time consuming (usually at least 2–3 days), which makes it difficult to use it for the pretreatment of immunoassays or MS analysis. Moreover, it is dependent on organic reagents and therefore risks not only protein denaturation but also environmental pollution. Hence, the development of more simple, effective and environment-friendly separation and purification techniques remains a challenging task.

As tropomyosin is a typical glycoprotein, boronate affinity may provide a novel and promising idea for its specific recognition and on-demand purification.⁹ Recently, phenylboronic acid (PBA) functionalized materials have attracted much attention in various biological applications, based on the specific and reversible covalent reaction between boronate affinity ligands and *cis*-diol-containing compounds.^{10,11} When the surrounding pH is greater than the pK_a value of the boronate affinity ligands, the boronic acid can effectively capture *cis*-diol-containing compounds and form five or six cyclic esters; then the boronic acid-*cis*-diol complex can be easily dissociated by adjusting the pH to acidic and so the

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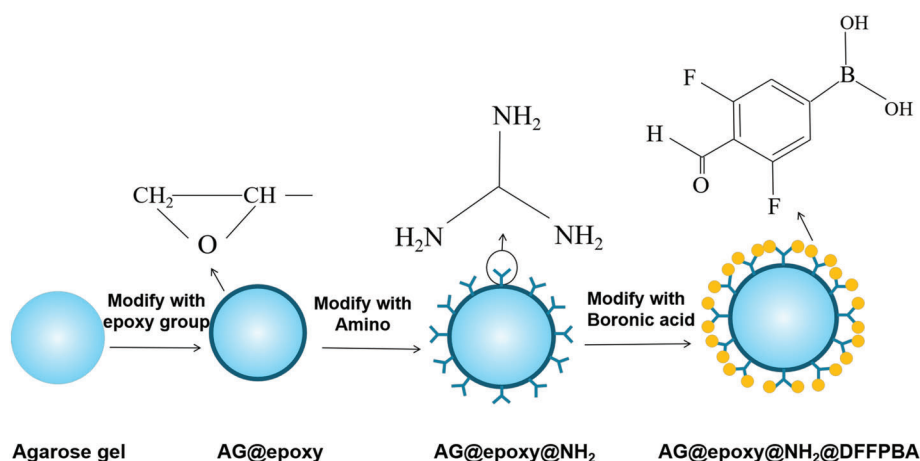


Figure 1. Schematic of the synthesis procedure of DFFPBA-agarose.

target *cis*-diol-containing compounds could be separated from the matrix. For separation and analysis of various *cis*-diol-containing compounds including glycoproteins, glycopeptides, nucleosides, and catechols, the technique has been demonstrated unique advantages in specificity, simplicity and economic efficiency in comparison to traditional approaches.^{12,13} Until now the application of boronate affinity for preparation of allergens has been very limited. Gu *et al.*¹⁴ enriched the major allergens of peanuts, milk, soybeans, and nuts in chocolate samples with a PBA-silica gel column. However, the separate purification of various allergens was not mentioned and the separation efficiency was not estimated. The high binding pH (pH = 11) may cause protein denaturation and may greatly limit its application in biological samples. Such a problem may be solved by the introduction of polysaccharide gums instead of rigid inorganic medium as the material matrix.¹⁵ For instance, agarose gel particles (AG) can usually provide much better biocompatibility along with satisfactory physical-chemical stability;^{16,17} moreover, their possible surface modification with 3,5-difluoro-4-formyl-phenylboronic acid (DFFPBA) and other new boronate ligands with both strong boronate affinity and relatively low pKa values (approximately 6.5)¹⁸ may greatly improve the isolation efficiency for proteins and other bio-molecules.

In this study, an agarose-based boronate affinity technique was developed for the first time for the purification of tropomyosin, based on a new strategy for the fabrication of affinity chromatography (Fig. 1): agarose gel particles were first modified with epoxy group, the tris(2-aminoethyl)amine was used as a dendrimer-assisted modification group, and the DFFPBA was introduced as a functional boronate affinity ligand. The developed technique was validated with *Paralichthys olivaceus*, *Thunnus thynnus*, *Oreochromis* spp. and *Lophius litulon*, and its efficiency was discussed in comparison with conventional methods.

MATERIALS AND METHODS

Reagents and apparatus

Agarose, Tris(2-aminoethyl)amine were purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). Epichlorohydrin was purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). 3,5-Difluoro-4-formyl-phenylboronic acid (DFFPBA) was purchased from Shanghai Yuanye Biological Technology Co., Ltd (Shanghai, China). SDS-PAGE Gel kit was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing,

China). Solid phase extraction (SPE) cartridges were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China). All other chemical reagents used were of analytical grade. *Paralichthys olivaceus*, *Thunnus thynnus*, and *Lophius litulon* were purchased from local markets in Qingdao, and *Oreochromis* spp. was purchased from a farm product market in Guangzhou.

Preparation of DFFPBA-Agarose gel particles

Fabrication of agarose gel particles were carried out according to the reverse embedding process¹⁹ with slight modification. Briefly, the oil phase consisting of 16 g of span-80 and 400 mL of paraffin oil was added into a 500 mL flask, followed by stirring at 24xg and heating to 80 °C in a water bath. Next, boiling 0.03 g mL⁻¹ agarose solution (2 g of agarose powder, 64 mL of deionized water) was dispersed slowly in the oil phase (400 mL) with continuous stirring at 80 °C for 30 min. Later, the reaction system was cooled to room temperature in an ice bath. The gel particles were collected and washed successively with 30 mL of petroleum ether, 30 mL of iso-propyl alcohol and 40 mL of deionized water. Finally, the agarose gel particles were stored in 200 mL L⁻¹ ethanol at 4 °C.

Agarose gel particles were epoxy-activated with epichlorohydrin. The mixed solution was shaken at 5 × g, which consisted of 5 g of agarose gel particles, 40 mL of epichlorohydrin, 50 mL of NaBH₄ solution (0.5 g L⁻¹) and 50 mL of NaOH solution (0.9 mol L⁻¹) at 40 °C. After the reaction, the epoxy-activated gel particles were washed with deionized water (5 × 15 mL) until the surplus amount of epichlorohydrin was removed completely. According to the previous report,²⁰ the final epoxy densities of agarose gels were determined by sodium thiosulfate titration. Briefly, 0.2 g of epoxy-activated gel particles, 3 mL of 1.3 mol L⁻¹ sodium thiosulfate, and 1–2 drops of phenolphthalein indicator were added into a flask, followed by shaking at room temperature for 30 min. After completion of the reaction, the mixture was titrated with 0.01 mol L⁻¹ hydrochloric acid standard solution until it changed from red to colorless. The epoxy density was calculated by Eqn (1), in which *S* is epoxy density (μmol g⁻¹), *C*_{HCl} is the concentration of hydrochloric acid standard solution (mol L⁻¹), *V*₀ and *V*₁ are the volume of HCl before and after titration (mL), *m* is the quality of epoxy-activated gel particles (g):

$$S = \frac{C_{\text{HCl}}(V_0 - V_1)}{m} \quad (1)$$

The epoxy-activated gel particles were mixed with 2 mL of tris(2-aminoethyl)amine and 20 mL of acetonitrile to modify them. This was stirred at 3×g and heated to 60 °C in a water bath for 12 h. Thereafter, modified agarose gel particles were washed twice with 40 mL of methanol and twice with 40 mL of acetonitrile.

Modified gel particles were added into the mixture of 20 mL of methanol, 300 mg of DFFPBA, and 600 mg of sodium cyanoborohydride in a 50 mL screw-capped vial, and reacted at room temperature for 72 h. Then the gel particles were washed with 40 mL of methanol, 20 mL of 0.5 g L⁻¹ NaHCO₃, 20 mL of 0.5 g L⁻¹ NaCl, and 30 mL of deionized water, respectively.

The morphology of DFFPBA-agarose gel particles was analyzed using scanning electron microscopy (SEM). This was conducted by the Affiliated Hospital of Qingdao with a Tescan 40 Vega3 (Tescan, Brno, Czech Republic). Surface functional groups were identified using a Nicolet iS10 Fourier-transform infrared (FTIR) (Thermo Fisher Scientific, Bruker, USA) in the wave number range of 400–4000 cm⁻¹ in attenuated total reflectance mode. The spectra were generated from an average of 40 scans acquired at a resolution of 4 cm⁻¹. DFFPBA-agarose gel particles and potassium bromide were mixed at a ratio of 1:7.

Pretreatment of fish samples

Fish muscle sample (2 g) was homogenized with 8 mL 0.01 mol L⁻¹ phosphate buffer saline (pH 7.4), and then allowed to stand still for 2 h. The mixture was then heated in a boiling water bath for 15 min and cooled down to room temperature, followed by centrifuging at 3823×g for 10 min to obtain the supernatant as a crude fish extract.

Procedures of the affinity chromatography

The prepared DFFPBA-agarose gel particles (1 mL) were transferred to a SPE cartridge (3 mL) with cover plates on both top and bottom. For affinity purification, the column was equilibrated at pH 7.4 with at least 10 column volume of buffer (0.01 mol L⁻¹ phosphate buffered saline). The 3 mL of crude fish extract sample was loaded into the affinity column and washed with the binding buffer at pH 7.4, followed by elution with 100 mmol L⁻¹ acetic acid (HAc). The eluted fractions were collected and their ultraviolet (UV) absorbance at 280 nm was determined by using a UV–visible spectrophotometer (Shanghai Qingpu Huxi Instrument Factory, Shanghai, China). All the fractions (unbound fractions, washed fractions, eluted fractions) were stored at 4 °C until they were further analyzed.

The protein concentrations of different samples and fractions were determined by a microplate reader (Molecular Devices, California, USA) using the Coomassie brilliant blue method and the protein purity of fractions was analyzed by regular SDS-PAGE (Bio-Rad Laboratories, California, USA) with 12% polyacrylamide gel and 5% stacking gel.²¹

To investigate the effect of agarose concentration on the efficiency of affinity chromatography, 0.96 g, 1.6 g, 2.0 g, and 2.5 g of agarose powder was dispersed thoroughly in 64 mL of deionized water, to form 0.015 g mL⁻¹, 0.025 g mL⁻¹, 0.03 g mL⁻¹, and 0.04 g mL⁻¹ agarose solution, respectively. Then, according to the method mentioned in the section of preparation of DFFPBA-Agarose gel particles, DFFPBA-agarose gel particles with different agarose concentrations were prepared and filled into columns for purification of tropomyosin in *Paralichthys olivaceus*.

To investigate the effect of binding pH on the efficiency of affinity chromatography, phosphate buffered saline (0.01 mol L⁻¹) was

prepared at pH 6.5, 7.0, 7.4, 8.0, and 9.0 and used for the extraction of *Paralichthys olivaceus* muscles as mentioned above. After extraction the pH values of crude extract was readjusted to 6.5, 7.0, 7.4, 8.0, and 9.0 with a trace amount of sodium hydroxide solution and phosphoric acid solution. Then the crude fish extract samples were loaded into the DFFPBA-agarose column (with 1 mL agarose) and washed with the corresponding binding buffers for purification.

The effect of ionic strength on the performance of the affinity chromatography was investigated by changing the concentration of NaCl in crude *Paralichthys olivaceus* extracts and binding buffer (0.01 mol L⁻¹ phosphate buffered saline, PBS) from 0.0 mol L⁻¹ to 0.7 mol L⁻¹. The crude fish extract was loaded into a DFFPBA-agarose column and washed by the binding buffers with different ionic strength, followed by elution with 100 mmol L⁻¹ HAc.

To investigate the effect of the elution condition on the efficiency of the affinity chromatography, the crude *Paralichthys olivaceus* extract was loaded into DFFPBA-agarose columns (with agarose gel particle of 1 mL) and washed with the binding buffer at pH 7.4, and then elution buffers (HAc) with different concentrations (25 mmol L⁻¹, 75 mmol L⁻¹, 100 mmol L⁻¹, 125 mmol L⁻¹, 150 mmol L⁻¹) to elute the tropomyosin.

In previous studies²² the enrichment efficiency of the affinity chromatography was calculated as follows:

$$R = \frac{E_0}{E} \times 100\% \quad (2)$$

where R (%) is enrichment efficiency of tropomyosin, E_0 (mg) is the tropomyosin content in the eluted fractions, and E (mg) is the tropomyosin content in the crude fish extract.

RESULTS AND DISCUSSION

Preparation and characterization of DFFPBA-Agarose gel particles

The epoxy density, always being associated with the density of affinity groups,^{23–25} was considered as one of the most important parameters for the modification of agarose gel to reach a high adsorption capacity and efficient purification.²⁶ Here, several main influences including the concentration of NaOH, NaBH₄, epichlorohydrin, and the reaction time, were investigated, and the highest epoxy density of agarose gels was determined as 151.74 μmol g⁻¹ under the optimized conditions of 0.5 g L⁻¹ NaBH₄, 0.9 mol L⁻¹ NaOH, 400 mL L⁻¹ epichlorohydrin, and 4 h reaction time (Fig. 2).

A scanning electron microscopy (SEM) image showed uniformly distributed DFFPBA-agarose gel particles (Fig. 3(a)), and no obvious crack was observed, which indicated the successful preparation of the agarose gel particles. A random selection of 200 microspheres from the SEM image for particle size measurement yielded a particle size distribution was in range of 40–160 μm. The reason for the relatively large particle size range may be artificial performance. Moreover, compared to initial gel particles, the gel surface would get tough after the modification, which will increase the irregular surface area for the interaction in application. This may be another cause of the above deviation.

After modification, the relative mass of boron in DFFPBA-agarose gel particles was calculated as 4.74% (m/m) according to the elemental analysis (Fig. 3(b)). The FTIR spectra shown in Fig. 3c provided another proof for the successful functionalization of DFFPBA on the surface of agarose gel particles. In spectrum (a), the weak IR peak at 3436 cm⁻¹ and the strong band at 2900 cm⁻¹

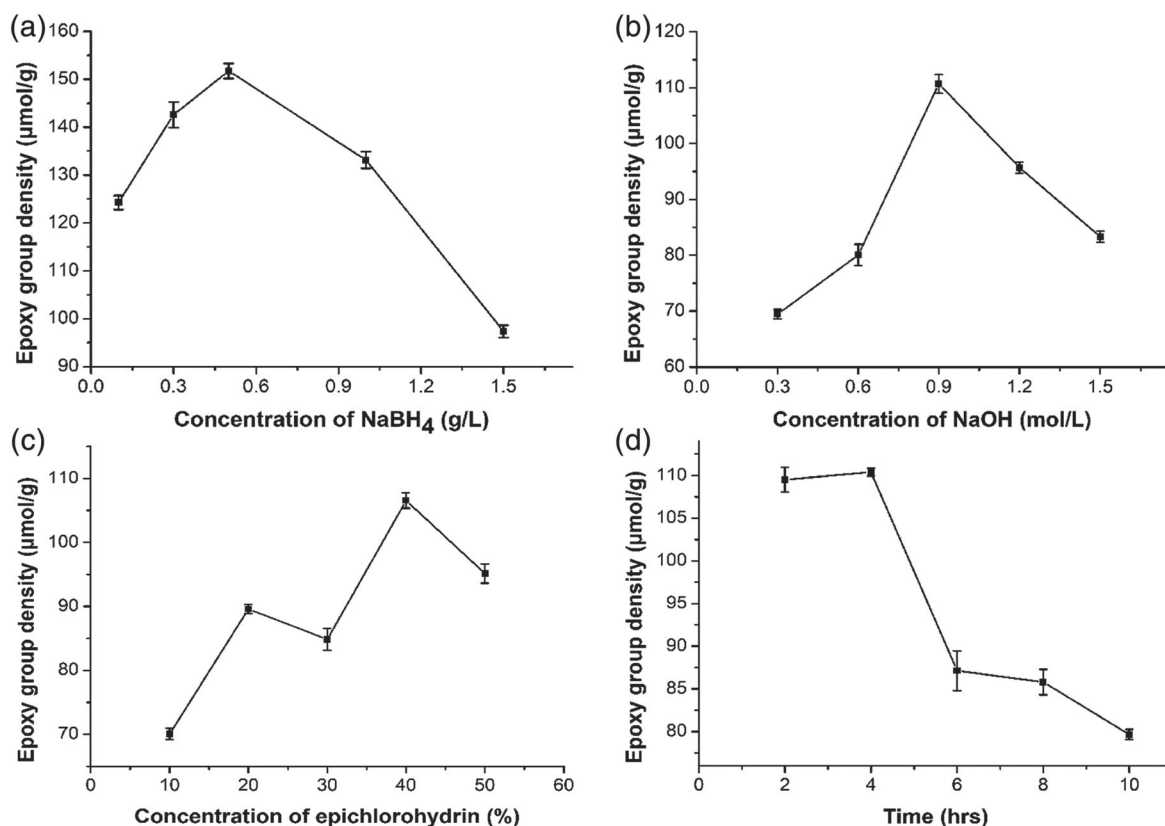


Figure 2. Optimization of agarose gel particle epoxidation conditions. Effects caused by the concentration of (a) NaBH₄, (b) NaOH, (c) epichlorohydrin, and (d) the reaction time.

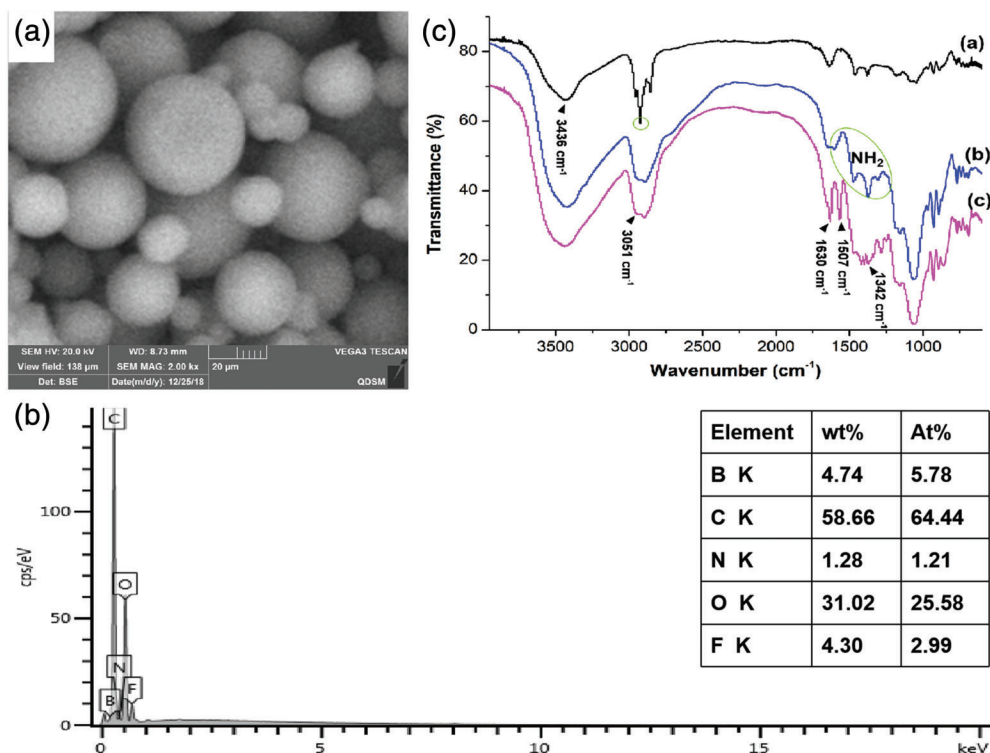


Figure 3. Characterization of DFFPBA-agarose gel particles. (a) SEM image of DFFPBA-agarose gel particles, (b) energy-dispersive X-ray spectra of DFFPBA-agarose gel particles, and (c) FTIR spectra of DFFPBA-agarose gel particles. The plots (a), (b) and (c) represent spectra of agarose gel particles, AG@NH₂, AG@NH₂@DFFPBA, respectively.

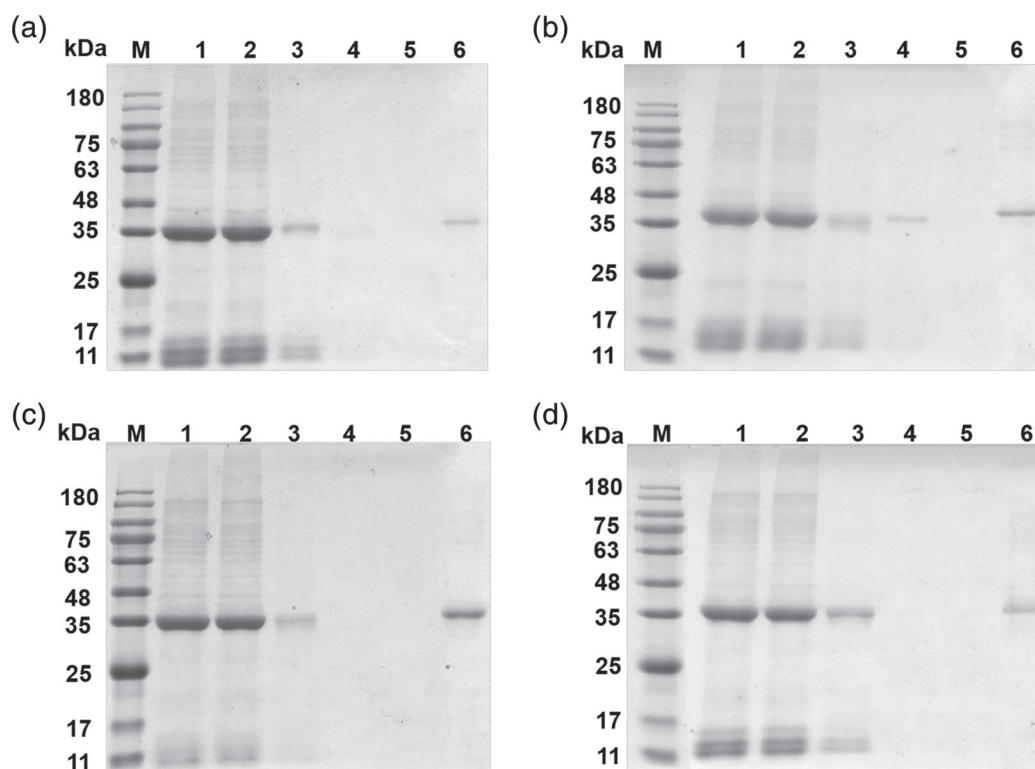


Figure 4. SDS-PAGE images of tropomyosin (36 kDa) purification by different agarose concentration. Tropomyosin purification by (a) 0.015 g mL⁻¹, (b) 0.025 g mL⁻¹, (c) 0.03 g mL⁻¹, and (d) 0.04 g mL⁻¹ agarose solution; M: protein molecular weight marker, Lane 1: crude fish extract, Lane 2: unbound fraction, Lane 3–5: washed fractions, Lane 6: eluted fraction.

could be assigned to the -O-H vibrations and saturated -C-H stretching vibration of agarose gel particles, respectively. The FTIR spectrum (b) of amino-agarose gel particles displayed bands at 1340–1559 cm⁻¹, which could be attributed to the bending vibration of the NH₂ in-plane. These results indicated that agarose gel particles were successfully wrapped by the -NH₂ groups. In spectrum (c), the effective immobilization of DFFPBA on the agarose gel particles was confirmed by the skeletal vibration (1500–1650 cm⁻¹), the stretching vibration of C—H of aromatic hydrocarbon (3051 cm⁻¹), and the absorption band of B—O at 1342 cm⁻¹. There were still some amino residues on the surface of the modified particles that will provide -B-N- 'teamed affinity' to extend the application in a lower pH environment.^{27,28} Overall, we could therefore draw the conclusion that DFFPBA-agarose gel particles were successfully synthesized.

Establishment, preliminary optimization and evaluation of the DFFPBA-agarose-based affinity chromatography for tropomyosin

The concentration of agarose could significantly affect the exclusive inner structure and was therefore considered to be an important factor for agarose gel-based chromatography.²⁹ Samples of *Paralichthys olivaceus* were used, with agarose concentrations of 0.015 g mL⁻¹, 0.025 g mL⁻¹, 0.03 g mL⁻¹, and 0.04 g mL⁻¹. The purity of tropomyosin in the eluted fraction (Fig. 4, Lane 6, MW 36 kDa) was evaluated as approximately 95%, 93%, 92% and 86%, respectively, and the column adsorption capacity was approximately 1.56 mg mL⁻¹, 1.87 mg mL⁻¹, 1.92 mg mL⁻¹ and 1.77 mg mL⁻¹. With lower agarose concentrations, relatively small gel particles would be formed with increased surface area, and therefore more boronate affinity ligands could be

immobilized and their ability to capture and separate tropomyosin would be significantly enhanced.³⁰ Moreover, with an increase in the agarose concentration, the viscosity of aqueous phase would also increase, which would result in reduced dispersity of droplets, enlarged particle size, and poor homogeneity in the continuous phase.¹⁹ These may have an adverse effect on the molecular sieve and diminish the purification efficiency of the agarose gel particles. But due to the high column pressure, it was also found that the bound tropomyosin could not be eluted completely when the concentration of agarose solution was below 0.03 g mL⁻¹, which resulted in a corresponding reduction of column capacity. An agarose concentration of 0.03 g mL⁻¹ seemed to be optimal and was applied in subsequent experiments.

In most cases the reversible covalent reaction efficiency between boronate affinity ligands and *cis*-diol-containing compounds depends on the binding pH.³¹ In general, boronate affinity requires a solution pH higher than the pK_a of boronic acid.³² Here, with different phosphate buffered saline buffers at pH levels ranging from 6.5 to 9.0, the purity of tropomyosin in the eluted fractions (Fig. 5, Lane 6, MW 36 kDa) was approximately 81%, 82%, 89%, 66% and 54%, respectively, and the column adsorption capacity was approximately 1.70 mg mL⁻¹, 1.76 mg mL⁻¹, 1.88 mg mL⁻¹, 0.57 mg mL⁻¹, and 0.54 mg mL⁻¹, respectively. Overall a binding pH value of 7.4 was demonstrated to be optimal. The high pK_a value of traditional boronate affinity ligands would require an alkaline binding pH (8–9) and therefore may result in the poor adsorption capacity and degeneration of biological samples,³³ so some new materials with low pK_a value have been developed, such as Wulff-type boronic acid, improved Wulff-type boronic acid, heterocyclic boronic acids, and boronate ligands with electron-withdrawing groups.³⁴ 3,5-Difluoro-4-formylphenylboric

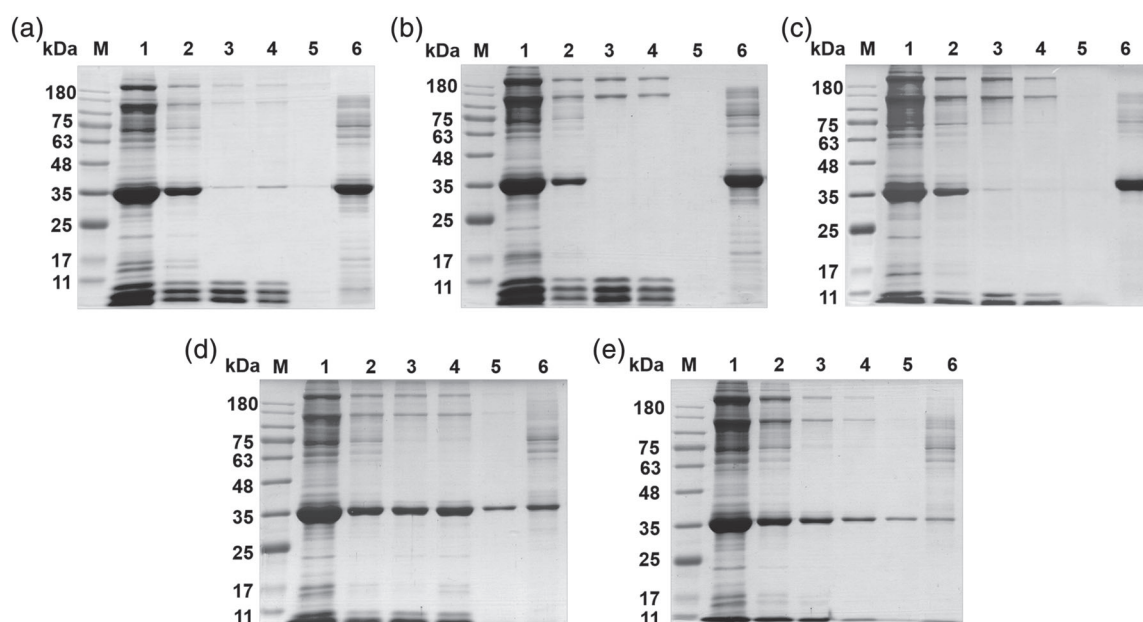


Figure 5. SDS-PAGE images of tropomyosin (36 kDa) purification at different binding pH values. Tropomyosin purification in (a) pH 6.5 binding buffer, (b) pH 7.0 binding buffer, (c) pH 7.4 binding buffer, (d) pH 8.0 binding buffer, (e) pH 9.0 binding buffer; M: protein molecular weight marker, Lane 1: crude fish extract, Lane 2: dilution of crude extract, Lane 3: unbound fraction, Lanes 4–5: washed fractions, Lane 6: eluted fraction.

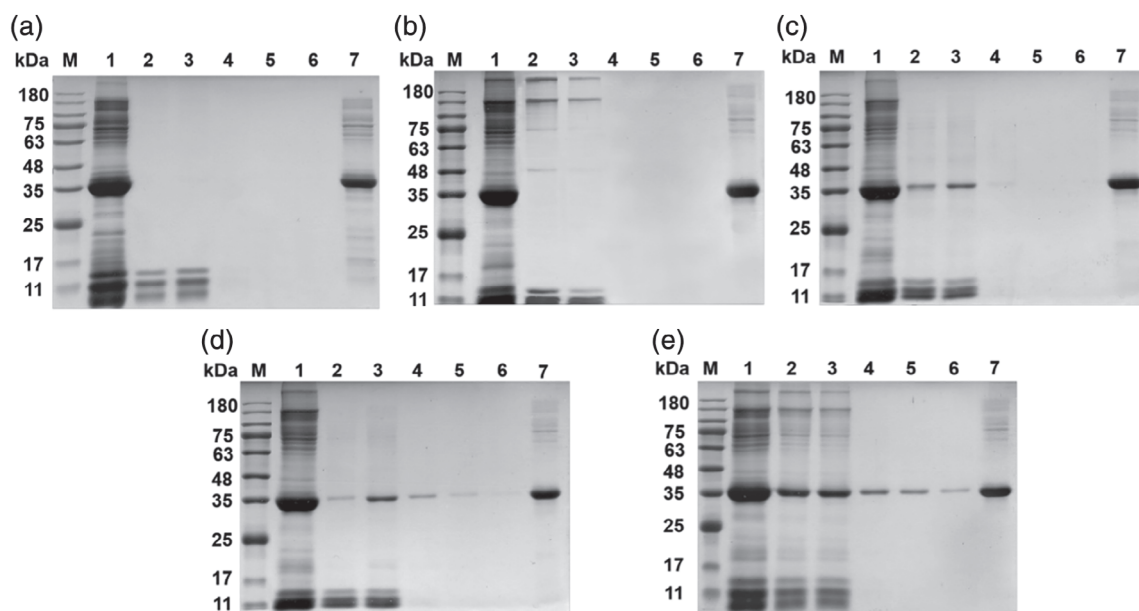


Figure 6. SDS-PAGE images of tropomyosin (36 kDa) purification in binding buffer (0.01 mol L⁻¹ phosphate, pH 7.4) containing different concentrations of NaCl. (a) binding buffer containing no NaCl, (b) binding buffer containing 0.1 mol L⁻¹ NaCl, (c) binding buffer containing 0.3 mol L⁻¹ NaCl, (d) binding buffer containing 0.5 mol L⁻¹ NaCl, (e) binding buffer containing 0.7 mol L⁻¹ NaCl; M: protein molecular weight marker, Lane 1: crude fish extract, Lane 2: unbound fraction, Lanes 3–6: washed fractions, Lane 7: eluted fraction.

acid had been proved to have high affinity at physiological pH environment, which is because the fluorine has strong electron absorption ability.^{35,36} Dendrimer-assisted modification was considered to be an effective method to enhance boronate affinity.³⁷ Here, to take the advantage of the two approaches, tris(2-aminoethyl)amine was used as the dendrimer-assisted modification group and 3,5-difluoro-4-formylphenylboronic acid was used as the affinity ligand. This strategy was validated well by the results. In the experiments, the increased isolation and purification efficiency with higher binding pH (from 6.5 to 7.4), (Fig. 5(a)–(c), Lane 6, MW 36 kDa) was also observed, and this

could be attributed to the enhancement of the specific boronate affinity.³⁸ However, when the pH was higher than 8.0, the purification efficiency decreased (Fig. 5(d) and (e), Lane 6, MW 36 kDa). This may be contributed to the complex interactions among multiple factors, including binding buffer, pKa of tropomyosin and affinity ligands.³⁹ For example, increased binding pH could promote the specific-affinity ability of boronate affinity ligands but may also result in their ionic repulsion with tropomyosin due to the negative charges.⁴⁰

With different concentrations of NaCl in the binding buffer (0.01 mol L⁻¹ PBS) from 0.0 mol L⁻¹ to 0.7 mol L⁻¹, the purities

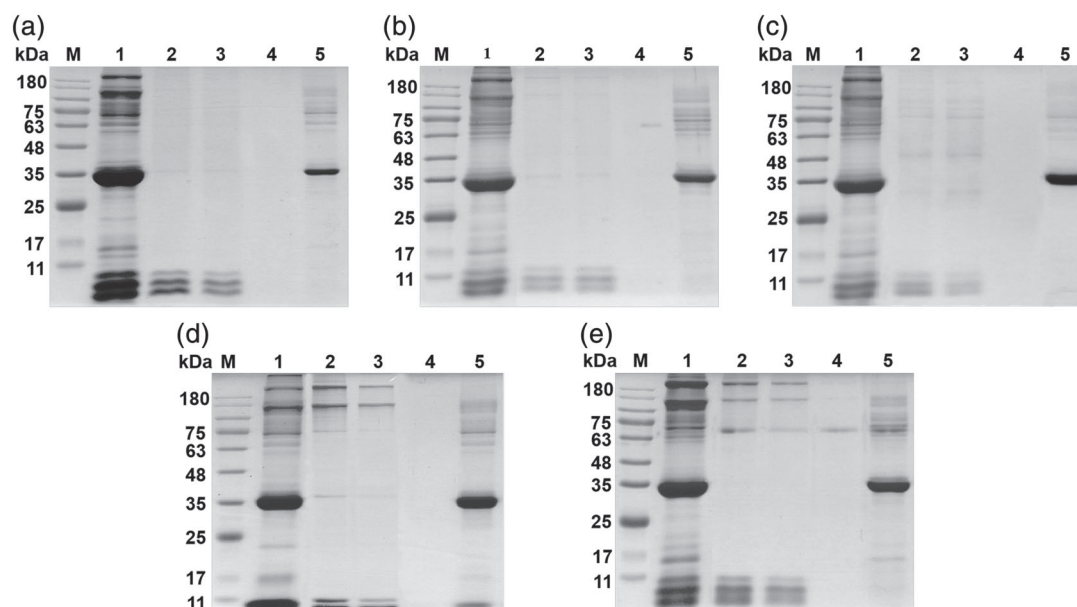


Figure 7. SDS-PAGE images of tropomyosin (36 kDa) purification in different concentration of elution buffer (HAc). Tropomyosin purification in (a) 25 mmol L⁻¹ elution buffer, (b) 75 mmol L⁻¹ elution buffer, (c) 100 mmol L⁻¹ elution buffer, (d) 125 mmol L⁻¹ elution buffer, (e) 150 mmol L⁻¹ elution buffer; M: protein molecular weight marker, Lane 1: crude fish extract, Lane 2: unbound fraction, Lanes 3–4: washed fractions, Lane 5: eluted fraction.

of tropomyosin in eluted fractions (Fig. 6, Lane 7, MW 36 kDa) were approximately 80%, 92%, 93%, 91%, and 88%, and the column adsorption capacities were approximately 1.87 mg mL⁻¹, 1.93 mg mL⁻¹, 1.42 mg mL⁻¹, 1.33 mg mL⁻¹, and 1.15 mg mL⁻¹, respectively. The increased tropomyosin purity with a higher NaCl concentration may be attributed to the reduction of non-specific adsorption. The boronate affinity ligands are negatively charged when the surrounding pH exceeds the pK_a value of the boronic acid, which will lead to an electrostatic interaction with basic proteins.^{41–43} It has been reported that the addition of a certain concentration of NaCl can effectively suppress such electrostatic interaction and reduce non-affinity adsorption.^{44,45} However, it was also found that the column capacity was decreased when the NaCl concentration was higher than 0.1 mol L⁻¹. This may be triggered by the hydrophobic effect of the column, which is associated with high ionic strength.⁴⁰ Considering the tropomyosin purity and column capacity, 0.1 mol L⁻¹ NaCl was therefore chosen as the proper salt concentration in the binding buffer and fish extracts.

Elution conditions also play a crucial role in boronate affinity chromatography.⁴⁶ With the concentration of HAc ranged from 25 mmol L⁻¹ to 150 mmol L⁻¹, the tropomyosin purities in the eluted fraction (Fig. 7, Lane 5, MW 36 kDa) were approximately 89%, 88%, 93%, 86%, and 83%, and the corresponding column adsorption capacity was approximately 1.01 mg mL⁻¹, 1.31 mg mL⁻¹, 1.94 mg mL⁻¹, 1.87 mg mL⁻¹, and 1.91 mg mL⁻¹ respectively. With the higher HAc concentration, the pH value of the elution buffer changed slightly, from pH 3.17 to pH 2.78, while the ionic strength changed from 0.025 to 0.15. Therefore, besides the boronate affinity, electrostatic interaction should be considered as another factor. When the HAc concentration was higher than 100 mmol L⁻¹, such electrostatic interaction could be greatly diminished with significantly increased ionic strength, and therefore the tropomyosin and other proteins would be eluted much more effectively. Such a result was consistent with the result from the investigation of the binding procedure. Taking both the elution amount and purity of tropomyosin into consideration,

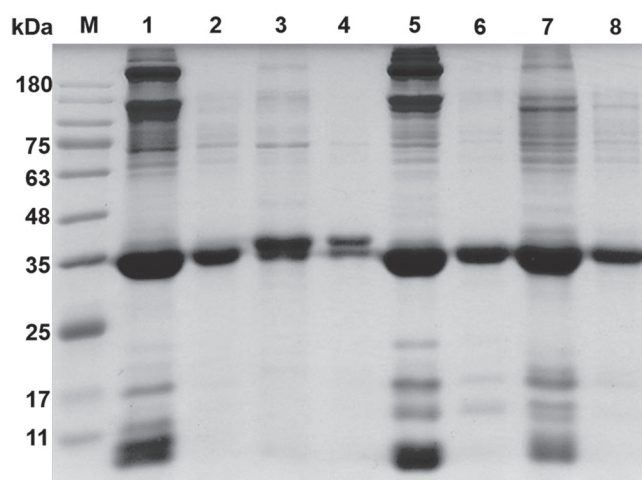


Figure 8. SDS-PAGE images of tropomyosin (36 kDa) purification of *Paralichthys olivaceus*, *Thunnus thynnus*, *Oreochromis* spp. and *Lophius litulon* using DFFPBA-agarose-based affinity chromatography. M: protein molecular weight marker, Lane 1: crude extract of *Paralichthys olivaceus*, Lane 2: eluted fraction of *Paralichthys olivaceus*, Lane 3: crude extract of *Thunnus thynnus*, Lane 4: eluted fraction of *Thunnus thynnus*, Lane 5: crude extract of *Oreochromis* spp., Lane 6: eluted fraction of *Oreochromis* spp., Lane 7: crude extract of *Lophius litulon*, Lane 8: eluted fraction of *Lophius litulon*.

100 mmol L⁻¹ was the most suitable concentration of HAc. Higher HAc elution may be applied to wash the column for regeneration.

Validation with different fish samples

The efficiency of the established DFFPBA-agarose-based affinity chromatography was validated with four different fish samples, including *Paralichthys olivaceus*, *Thunnus thynnus*, *Oreochromis* spp., and *Lophius litulon*. The tropomyosin purity in the eluted fraction (Fig. 8) was approximately 93%, 95%, 89%, and 91%; the column adsorption capacity was approximately 1.95 mg mL⁻¹,

Table 1. Summary of tropomyosin purification methods

Research object	Purification steps	Time	Whether to use organic reagents	Purity	Reference
<i>Kuruma Prawn</i>	extraction with KCl/NaHCO ₃ buffer→ washing with ethanol and diethyl- ether→ overnight extraction with tris-HCl → two times of isoelectric point precipitation→ ammonium sulfate fractionation	2 days	yes	Approx. 98%	47
<i>Penaeus vannamei</i>	extraction with buffer solution→ four times of extraction with buffer A (50 mmol L ⁻¹ KCl and 2 mmol L ⁻¹ NaHCO ₃) and buffer B (20 mmol L ⁻¹ Tris-HCl, 1 mol L ⁻¹ KCl and 10 mmol L ⁻¹ DTT) → washing with acetone→ ammonium sulfate fractionation	1–2 days	yes	Approx. 93%	48
<i>Procambarus clarkii</i>	four times of extraction with buffer A (10 mmol L ⁻¹ KCl, 20 mmol L ⁻¹ NaHCO ₃ and 10 mmol L ⁻¹ EDTA) → dissolution with acetone→ extraction with buffer B (20 mmol L ⁻¹ Tris-HCl, pH = 7.5, 1 mol L ⁻¹ KCl and 10 mmol L ⁻¹ DTT) → isoelectric point precipitation→ ammonium sulfate fractionation→ heat treatment	1–2 days	yes	Approx. 95%	49
<i>Octopus fangsiao</i>	crude extraction with ice-cold 20 mmol L ⁻¹ phosphate buffer (pH 7.4) for four times→ isoelectric precipitation→ ammonium sulfate fractionation→ purification with DEAE-sepharose column	1–2 days	No	Above 90%	50
<i>American lobster, Japanese flying squid and turban shell</i>	extraction with 0.01 mol L ⁻¹ phosphate buffer (pH 7.0) → heat treatment→ extraction with acetone→ extraction with 25 mmol L ⁻¹ tris-HCl buffer (pH 8.0, containing 1 mol L ⁻¹ KCl, 0.1 mmol L ⁻¹ CaCl ₂ and 1 mmol L ⁻¹ dithiothreitol) → ammonium sulfate fractionation→ purification with reverse-phase HPLC (TSK gel ODS-120 T column)	2–3 days	yes	Above 90%	51
<i>Freshwater shrimps</i>	extraction with acetone→ overnight extraction with phosphate buffer→ ammonium sulfate fractionation→ purification with G-50 gel column→ purification with DEAE-ion exchange column	3–4 days	yes	94.2%	52
This work (<i>Paralichthys olivaceus</i> , <i>Thunnus thynnus</i> , <i>Oreochromis spp.</i> , and <i>Lophius litulon</i>)	extraction with 0.01 mol L ⁻¹ phosphate buffer saline (pH 7.4) → heat treatment→ purification with DFFPBA-agarose-based affinity chromatography	3–4 h	No	Above 90%	/

1.88 mg mL⁻¹, 1.93 mg mL⁻¹ and 1.91 mg mL⁻¹, and the column enrichment efficiency of tropomyosin was approximately 65%, 73%, 67%, and 66%, respectively. Compared with previous reported methods (Table 1), the novel technique established here could be applied to a variety of fish samples with similar tropomyosin purity. This could result in significantly decreased performance time (from at least 1–2 days to 3–4 h), much simplified procedures (from at least 5 steps to 3 steps), and excellent biocompatibility (without the introduction of any organic solvent in purification process).

The affinity ligands prepared here did not exhibit the ability to recognize parvalbumin (12 kDa), which is also a kind of glycoprotein. Possible reasons may include: (1) Boronate affinity ligands specifically interact with glycoproteins by recognizing the oligosaccharides. In comparison with tropomyosin, which contains much more column adsorption capacity over 1.85 mg mL⁻¹ (4.4%–6.9%),⁵³ parvalbumin only has a single glucose residue on the cysteine at the amino acid sequence at position 18, so the oligosaccharide content is predicted not

more than 1.8% (m/m).⁵⁴ Thus, in principle it has little chance to compete with tropomyosin for affinity ligands. (2) The oligosaccharides of parvalbumin may not be exposed very well due to the spatial structure of parvalbumin, which reduces the efficiency of boronate affinity ligand-oligosaccharide interaction. (3) The agarose gel particles may also have a certain ability to separate different glycoproteins based on the principle of molecular sieve.⁵⁵ With more precise design of boronate affinity ligands and chromatographic performance, multiple affinity performances, or in combination with other techniques such as molecular sieve and charge transfer interactions,⁴⁵ such a significant difference in oligosaccharide content may be also used for the effective isolation of parvalbumin and other glycoproteins.

There were still a few non-targeted proteins in the eluted fractions. They may have been some kind of glycoprotein, besides parvalbumin and tropomyosin in the fishes, or some matrix proteins non-specifically interacting with the DFFPBA-agarose gel particles. Through further optimization of sample pretreatments and introduction of new elution strategies, such as competitive elution,

one can expect more effective elimination of these impurities and therefore further improvement in tropomyosin purity.

CONCLUSIONS

In this paper, a new boronate affinity chromatography was produced by using DFFPBA as a functional monomer, tris(2-aminoethyl)amine as a multi-branched ligand, and agarose gel particles as supporting materials. Then this was applied for the first time for the isolation of tropomyosin, and its efficiency was validated with different fish samples. With an agarose concentration of 0.03 g mL^{-1} , binding pH at 7.4, and HAc concentration of 100 mmol L^{-1} , the estimated tropomyosin purity was higher than 90%, the column adsorption capacity was examined over 1.85 mg mL^{-1} , and the tropomyosin enrichment efficiency of column was above 65%. In comparison with traditional methods, the established technique demonstrated a significant improvement in rapidity and simplicity, which indicated its promising application for the isolation of tropomyosin and other allergens in food stuffs.

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