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Short communication

Trypsin purification using magnetic particles of azocasein-iron composite

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ABSTRACT

This work presents an inexpensive, simple and fast procedure to purify trypsin based on affinity binding with ferromagnetic particles of azocasein composite (mAzo). Crude extract was obtained from intestines of fish Nile tilapia (*Oreochromis niloticus*) homogenized in buffer (01 g tissue/ml). This extract was exposed to 100 mg of mAzo and washed to remove unbound proteins by magnetic field. Trypsin was leached off under high ionic strength (3 M NaCl). Preparation was achieved containing specific activity about 60 times higher than that of the crude extract. SDS-PAGE showed that the purified protein had molecular weight (24 kDa) in concordance with the literature for the Nile tilapia trypsin. The mAzo composite can be reused and applied to purify trypsin from other sources.

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1. Introduction

The use of magnetic particles in biotechnology has been widely used as a result of their applications in various fields in biomedicine, diagnostic and as the food industry. This technique is an alternative for the immobilization and purification of biomolecules, making the process more economical, fast, stable and creating a high quality product (Cao et al., 2012; Coelho et al., 2016; Maciel et al., 2012).

Fish viscera contain a variety of applicable enzymes, especially proteases that are about 50% of total enzymes sold worldwide (Khangembam & Chakrabarti, 2015; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). Among them trypsin stands out for its importance in biotechnology (Khangembam & Chakrabarti, 2015).

Here it is presented a simple, fast and low cost process to purify trypsin based on magnetic particles of azocasein-iron composite. Trypsin purification from fish viscera was used as a purification example. Precisely, trypsin from tilapia (*Oreochromis niloticus*) viscera was purified. Azocasein is a nonspecific substrate of the proteases that consists of casein conjugated to an azo dye. This substrate is used for proteases assays because it is capable to be degraded under enzyme catalysis releasing soluble dye (peptide derivative) into the supernatant that can be quantitatively analyzed.

2. Material and methods

2.1. Reagents

Trypsin (E.C.3.4.21.4) from porcine pancreas, bovine serum albumin (BSA), *N*-benzoyl-D-L-arginine-*p*-nitroanilide (BAPNA), aniline (ACS agent), glutaraldehyde (25%) and azocasein were purchased from Sigma-Aldrich (USA). Dimethylsulfoxide (DMSO) was acquired from Vetec chemical (Brazil). Ferric chloride hexahydrate and ferrous chloride tetrahydrate were obtained from Merck (Germany). All other reagents were of analytical grade.

2.2. Preparation of magnetic composites

The procedure to obtain the magnetic particles of azocasein-iron composite (mAzo) was adapted from the protocol based on Carneiro Leão, Oliveira, and Carvalho (1991). Briefly, an aqueous mixture containing 10 mL of $FeCl_3{\cdot}6H_2O$ (1.1 M) and 10 mL of $FeCl_2{\cdot}4H_2O$ (0.6 M) was added to 100 mg of azocasein solution (prepared in 5 mL of Tris-HCl 0.1 M, pH 8.0). Ammonium hydroxide 28.0–30.0% was then added to achieve a pH of 11. The mixture was then heated up to 50 °C and kept for 30 min under vigorous stirring. The magnetic particles synthesized were thoroughly

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washed with distilled water to pH 7.0. The material was dried at 50 °C and kept at 25 °C for later use. Alternatively, magnetic particles were coated with polyaniline (mPANI), activated with glutaraldehyde and afterwards azocasein was covalent linked (mPANI-Azo) according to Neri et al. (2009).

2.3. Fish crude extract

Specimens of Tilapia (*Oreochromis niloticus*) were captured from the fishpond facilities at the Departamento de Pesca, Universidade Federal Rural de Pernambuco (Brazil). The fish intestines were collected and homogenized (1 mg of tissue mL^{-1}, w/v, in 5 mL of 0.01 M Tris-HCl buffer, pH 8.0) by using a tissue homogenizer (COLE-PARMER Model 04369-15). The resulting preparation was centrifuged at 10,000*g* for 25 min at 4 °C to remove fat. The supernatant (crude extract) was frozen at −20 °C and used for further purification steps.

2.4. Trypsin purification

Fish crude extract (2 mL) was incubated with either mAzo or mPANI-Azo (both 0.1 g) for 2 h at 4 °C under mild stirring. After this time, the particles were washed with 0.1 M Tris-HCl buffer, pH 8.0 seven times and 3 M NaCl three times. The first supernatant and washings were collected for protein (Bradford, 1976) and enzymatic activity (Amaral, Carneiro-da-Cunha, Carvalho Jr, & Bezerra, 2006) determinations.

2.5. Reuse of mAzo

The purification procedure above described was carried out four times using the same mAzo preparation. Supernatants and washings were also collected for protein and enzymatic activity determinations.

2.6. Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970), using a 6% (w/v) stacking gel and a 12.5% (w/v) separating gel. The gels were stained for protein in 0.01% (w/v) Coomassie Brilliant Blue (overnight). The background of the gel was destained by washing with hot distilled water.

3. Results and discussion

Firstly, mAzo (10 mg) was incubated with a commercial trypsin from porcine pancreas (1 mL containing 2 mg) and the purification procedure was carried out. A preparation containing 1.85 mg of protein and 1.24 U (0.067 U/mg), which was 4.53-fold purified compared to the commercial trypsin. These results demonstrated that mAzo was capable to bind the enzyme (affinity) and to release it under 3 M NaCl.

Table 1 shows the fish crude extract purification using mAzo (100 mg). A preparation of trypsin was obtained containing

Table 1
Purification of trypsin from tilapia with magnetite, mAzo and mPANI-Azo.

Sample	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude extract	11.81	2.20	0.2	100.0	1
Magnetite	0.10	0.04	0.4	1.8	2
mAzo	0.03	0.37	12.3	16.8	61.5
mPANI-Azo	0.04	0.35	8.7	15.9	43.5

All operations were carried out at 4 °C.

Table 2
Trypsin purification from fish viscera results.

Purification Method	Purification Fold	Source	Reference
Heating + $(NH_4)_2SO_4$ + Sephadex G75	51.1	*Colossoma macropomum*	Bezerra et al. (2001)
Heating + $(NH_4)_2SO_4$ + Sephadex G75	21.6	*Oreochromis niloticus*	Bezerra et al. (2005)
$(NH_4)_2SO_4$ + DEAE Sephacel + Sephacryl S-200	13	*Sardinops sagax caerulea*	Castillo-Yáñez, Pacheco-Aguilar, García-Carreño, and Navarrete-Del Toro (2005)
(NH4)2SO4 + SBTI-Sepharose 4B	34	*Priacanthus macracanthus*	Van Hau and Benjakul (2006)
$(NH_4)_2SO_4$ + Heated + Sephadex G-100	9.2	*Sardina pilchardus*	Bougatef, Souissi, Fakhfakh, Ellouz-Triki, and Nasri (2007)
$(NH_4)_2SO_4$ + Acetone + SBTI-Sepharose 4B	40.6	*Pomatomus saltatrix*	Klomklao et al. (2007)
Sephacryl S-200 + Sephadex G-50	59	*Sebastes schlegelii*	Kishimura et al. (2007)
Sephacryl S-200 + Sephadex G-50	32	*Alcichthys alcicornis*	Kishimura et al. (2007)
Sephacryl S-200 + Sephadex G-50	33	*Gadus macrocephalus*	Tomoyoshi Fuchise et al. (2009)
Sephacryl S-200 + Sephadex G-50	28	*Eleginus gracilis*	Tomoyoshi Fuchise et al. (2009)
Acetone + Sephadex G-100 + Mono Q	13.9	*Balistes capriscus*	Jellouli et al. (2009)
$(NH_4)_2SO_4$ + Sephadex G-100 + DEAE cellulose + Sephadex G-75 + Q-Sepharose	26.7	*Sepia officinalis*	Balti, Barkia, Bougatef, Ktari, and Nasri (2009)
Acetone + $(NH_4)_2SO_4$ + Sephacryl S-200 + DEAE-Sephacel	11.2	*Oreochromis niloticus X O. aureus*	Wang et al. (2010)
$(NH_4)_2SO_4$ + Sephadex G-100 + Mono Q Sepharose + Ultrafiltration	4.2	*Salaria basilisca*	Ktari et al. (2012)
$(NH_4)_2SO_4$ + Sephadex G-100 + Mono Q	27.1	*Barbus callensis*	Sila et al. (2012)
$(NH_4)_2SO_4$ + SBTI-Sepharose 4B	26.4	*Aluterus monóceros*	Zamani and Benjakul (2016)
$(NH_4)_2SO_4$ + Dialysis + DEAE cellulose + Benzamidine column	35.6	*Cirrinus mrigala*	Khangembam and Chakrabarti (2015)
mAzo	61.5	*Oreochromis niloticus*	This work
mPANI-Azo	43.5	*Oreochromis niloticus*	This work

Table 3
Reuse of mAzo particles.

Use	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude extract	11.81	2.20	0.2	100	1
1st	0.02	0.23	11.5	10.45	57
2nd	0.02	0.22	11	10	55
3rd	0.02	0.23	11.5	10.45	57
4th	0.02	0.20	10	9.10	50

All operations were carried out at 4 °C.

0.03 mg of protein with 0.37 U (12.3 U/mg), which was 61.5-fold purified compared to the crude extract. It is also shows that the magnetic particles without azocasein (Magnetite) yield a preparation 2 times purified, namely, negligible purification was achieved. The mPANI-Azo demonstrated that trypsin was also purified from fish crude extract resulting a preparation containing specific activity similar to that attained using mAzo. However, the synthesis of mPANI-Azo involves more steps than mAzo.

The theoretical basis of the present procedure is the presence of azocasein on the surface of the magnetite that would allow the affinity bind trypsin molecules presents in the crude extract. The attraction of the particles (mAzo-trypsin complex) by magnetic field would remove it from the mixture. Finally the high ionic strength (3 M NaCl), would disrupt the complex releasing the soluble trypsin and insoluble mAzo that is attracted by the magnetic field. This collected mAzo could be reused. The decrease of azocasein degree freedom attached to magnetic particles would allow the complex formation with trypsin but not the substrate hydrolysis. It is important to register that no colored product (azopeptides) was detected from the mAzo-Trypsin complex even after 24 h of incubation. The covalent azocasein magnetite derivative (mPANI-Azo) would act in the same way, but its synthesis involves four steps.

Table 2 shows methods for trypsin purification from fish viscera reported in the literature. The present method showed better performance than those reported for tilapia (Bezerra et al., 2005; Wang et al., 2010) with the advantage of involving one step only. Furthermore, the purification fold showed to be higher than those reported for other fish species.

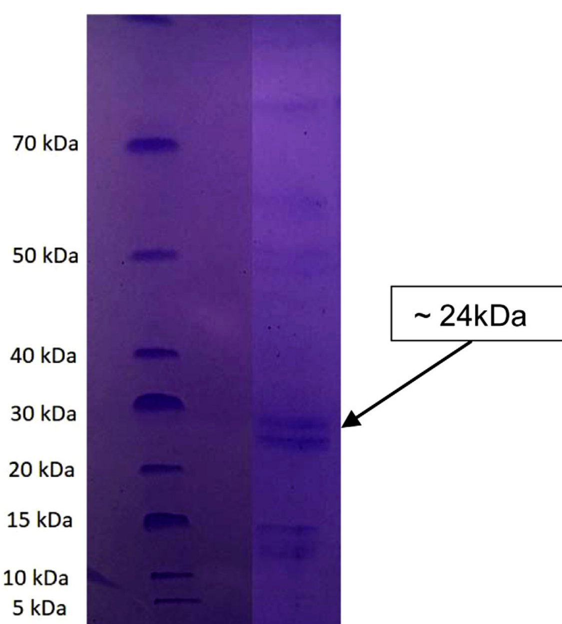

Fig. 1. SDS-PAGE trypsin from tilapia purified after mAzo exposure, compared to standard proteins.

Table 3 shows the reuse of the same mAzo preparation to purify trypsin from fish crude extract four times. A small decrease of efficiency was observed either expressed in terms of yield or purification fold, respectively, from 10.45% to 9.1% and from 57-fold to 50-fold. It is worthwhile to register that mAzo showed to stable at 25 °C for one year.

SDS-PAGE of the purified enzyme by the mAzo procedure showed two bands of protein (Fig. 1) with molecular weight about 24 kDa, value reported for tilapia trypsin (Bezerra et al., 2005). The molecular weight of trypsin from other fish viscera has been reported inside the range of 20.0–30.0 kDa (Khangembam & Chakrabarti, 2015).

4. Conclusion

This contribution presents an inexpensive, simple and fast one step procedure to purify trypsin from fish viscera based on affinity binding onto ferromagnetic azocasein composite. Azocasein covalently linked to ferromagnetic particles coated with polyaniline also showed to be capable to bind trypsin. However, this last composite has the disadvantage of involving four steps during the synthesis. The reuse of the ferromagnetic azocasein composite is another advantage of this proposal that showed promising application compared to the methods reported in the literature. This procedure can be applied for trypsin purification from other sources.

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There is no conflict of interest.

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