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Immobilized protease on magnetic particles for enzymatic protein hydrolysis of poultry by-products

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ABSTRACT

Immobilization of enzymes onto magnetic particles can potentially allow for enzyme reuse, which can significantly reduce the cost associated with e.g. enzymatic protein hydrolysis (EPH) of fish and meat by-products. Here, we report glutaraldehyde-mediated immobilization of a food-grade protease (Subtilisin A) onto magnetic silica particles using three different amine ligands; a short, brush-like linker (aminopropyl trimethoxysilane), a long, flexible linker (Jeffamine), and a gel-like coating (chitosan). The three coupling strategies were evaluated and compared with respect to the amount of immobilized protease, enzyme activity and catalytic performance in the hydrolysis of chicken meat and turkey tendons, respectively. The particle systems showed high reusability ($\leq$85% activity remaining after six consecutive cycles) and storage stability ($\leq$93% activity remaining after 25 months storage). Particle-immobilized enzyme systems were able to catalyze degradation and extraction of protein from chicken meat and turkey tendons.

1. Introduction

Immobilization of enzymes – both on solid supports and on suspended particles - is an acknowledged method in many fields of biocatalysis for stabilizing enzymes in harsh production environments encompassing e.g. high temperature, pressure, and organic solvents (Ansari & Husain, 2012; Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). In the special case of proteases, a possible additional benefit is the prevention of autolysis (Garcia-Galan et al., 2011). Immobilization can also be used to modify enzyme activity and selectivity – see e.g. the excellent tutorial review by Rodrigues et al. (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernandez-Lafuente, 2013). As a detailed description of enzyme immobilization and the associated advantages and drawbacks is beyond the scope of the present paper, we recommend several excellent reviews (Barbosa et al., 2015; Garcia-Galan et al., 2011; Rodrigues et al., 2013) on the subject.

After carboxylases, proteases are the second largest industrial enzyme market. One industrial application of proteases is in enzymatic protein hydrolysis (EPH) of by-products, where food-grade proteases are used to perform proteolysis on e.g. fish and meat by-products in valorization towards e.g. food, feed, pet food and functional food applications (Aspevik et al., 2017). In the proteolysis of these complex raw materials, the cost related to protease addition is one of the most important economic variables (Aspevik, Egede-Nissen, & Oterhals, 2016). Moreover, the cost of industrial proteases for EPH varies greatly. A comparison performed in the fall of 2019 showed that the cost of 1 kg commercial food grade protease from 10 different vendors varied between £11 and £125 (Solstad et al., 2020). Thus, with the aim to develop a cost-efficient process, it is reasonable to assume that the choice of protease will be influenced by the cost. Hence, the possibility to immobilize proteases for enzyme reuse with a concomitant cost reduction becomes attractive. Furthermore, some commercial proteases such as Alcalase 2.4 L, have also been reported to suffer from both product inhibition, thermal inactivation and autolysis, limiting product yield (Apar & Özbek, 2008; Colleary & Fágáin, 2008; Valencia, Pinto, & Almonacid, 2014). Hence, immobilization is also interesting from the perspective of reducing such undesired process-limiting properties.

During industrial EPH of by-products, proteases are added to an aqueous mixture (consisting of 1:2 to 1:1 ratios of minced by-products to water) and run at a temperature of 50–60 °C in batch or continuous reactors. These by-products often contain high amounts of fat, tendons and bones (Wubshet et al., 2018). Due to the proteolysis and the

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elevated temperatures during EPH, the water-phase gradually becomes mixed with considerable amounts of melted fats and liberated particular matter, as well as remaining undigested by-products. After the hydrolysis reaction is finished, the proteases are generally inactivated by heating. This also serves as a pasteurization step. To separate the protein-rich water phase from the fat and sediment fractions in this highly heterogeneous mixture at an industrial scale, a sieve is oftentimes used to separate remaining bones followed by centrifugation by a de-/tricanter (Pasupuleti & Demain, 2010; Vang et al., 2018, pp. 459–476).

Alcalase 2.4 L, the brand name of a protease product containing primarily Subtilisin A from Bacillus licheniformis, is a recognized protease for EPH of marine and animal-based by-products, and as such, has been subjected to a number immobilization studies. Alcalase immobilization strategies have been directed both towards industries with reactions performed at high temperatures and/or in the presence of e.g. organic solvents (Bernal, Guzman, Illanes, & Wilson, 2018; Chen, Hsiao, Chiou, Wu, & Wang, 1992; Vossenberg et al., 2013; Wang et al., 2014), and for industries performing proteolysis with water-soluble proteins (da Cruz et al., 2020; Pessato et al., 2016; Sousa Jr et al., 2004; Tardioli, Pedroche, Giordano, Fernández-Lafuente, & Guisán, 2003; Wang et al., 2014).

An immobilization strategy which could allow for reuse of enzymes is the immobilization onto magnetic particles, where a magnetic field can separate the particles from the reaction mixture after the enzymatic action has taken place. Use of magnetic particles in complex, high-viscous media such as used in industrial hydrolysis of waste streams is still a very challenging task. Specifically, a unique set of challenges for any industrially relevant immobilization strategy aimed at the EPH industry handling animal-based by-products is the highly heterogeneous reaction mixture and the separation steps, especially when aiming to reuse the immobilized proteases. Consequently, studies of hydrolysis reactions using particle-immobilized enzymes are typically done on simple protein solutions such as casein, as demonstrated by Aslani and coworkers (Aslani, Abri, & Pazhang, 2018) The successful design of magnetic particles as enzyme scaffolds for industrial hydrolysis requires a careful balance between particle size, magnetic core properties (typically iron oxides), particle morphology and porosity, colloidal stability and functionalization. Specifically, the particles should allow for rapid separation in the complex fluid when a magnetic field is applied, while also allowing for sufficient colloidal stability and a high surface area for enzyme immobilization. Using porous as opposed to non-porous magnetic particles is an established method for increasing enzyme loading and thus total theoretical activity, as e.g. demonstrated by Feng et al. (Feng et al., 2021). However, it should be noted that using porous particles for enzyme immobilization might lead to attenuated net contact between enzyme and substrate, especially for complex reaction mixtures such as meat and tendons, which cannot access the pores. The coupling strategy used for enzyme immobilization is a pivotal component for success, as it should allow for maximum enzyme coverage without surface-induced denaturation or steric hindrance. Here, we have elucidated the effect of coupling strategy by glutaraldehyde-mediated immobilization of a food-grade protease (Subtilisin A) onto magnetic silica particles using three different amine ligands; a short, brush-like linker (aminopropyl trimethoxysilane), a long, flexible linker (Jeffamine), and a gel-like coating (chitosan). The three coupling strategies were initially evaluated and compared with respect to the amount of immobilized protease and enzyme activity. Subsequently, a proof-of concept study was performed where the particles were used to catalyze hydrolysis of chicken meat and turkey tendons. The two raw materials were chosen to provide two test systems ranging from easy to digest muscle myofibril protein (*i.e.;* chicken muscle) to hard to hydrolyze connective tissue (*i.e.;* turkey tendon). Thus, the novelty of the present study comes from applying robust and scalable covalent coupling chemistry to magnetic silica particles functionalized with different surface morphologies and assessing the resulting catalytic performance using complex reaction mixtures.

2. Materials and methods

2.1. Materials

Aminopropyl trimethoxysilane (ATMS), 3-glycidyloxypropyl trimethoxysilane, glutaraldehyde, phosphate buffer (pH 7.0 and 7.7), acetic acid, trichloroacetic acid, Bradford reagent B6916, ninhydrin, dimethyl sulfoxide (DMSO), Span80, toluene, methanol, nitric acid, lithium hydroxide (LiOH), tin (II) chloride (SnCl$_2$), chitosan (medium molecular weight, 190–310 kDa), protease from *Bacillus licheniformis* P5380 (Subtilisin A) and azo-casein were all purchased from Sigma Aldrich (St. Louis, Missouri, USA). JEFFAMINE D-400 (average molecular weight 430 g/mol) was purchased from Huntsman Performance Products (The Woodlands, Texas, USA). Ferromagnetic magnetite particles were purchased from Ferrotec (Santa Clara, California, USA), and waterglass (sodium silicate solution) was purchased from Norsk Medisinaldepot (Trondheim, Norway). Analytical grade acetonitrile, trifluoroacetic acid, monosodium phosphate and molecular weight standards (bovine serum albumin, heart, aprotinin from bovine lung, insulin chain B oxidized from bovine pancreas, renin substrate tetradecapeptide porcine, angiotensin II human, bradykinin fragment 1–7, [DAla2]-leucine encephalin, Val-Tyr-Val and tryptophan) were purchased from Sigma-Aldrich (St. Louis, MO). Water used for HPLC was purified by deionization and 0.22 mm membrane filtration (Millipore, Billerica, Massachusetts, USA).

2.2. Methods

2.2.1. Preparation of pH 5.2 lithium acetate buffer

The pH 5.2 lithium acetate buffer was prepared as follows: 9.8 g of LiOH was dissolved in 40 mL deionized (DI) water under vigorous stirring. When approximately half of the added salt was dissolved, 29.3 mL of acetic acid was added (Note! Exothermic reaction). The mixture was then stirred and allowed to cool to room temperature, and DI water was added to yield a total volume of 100 mL.

2.2.2. Preparation of magnetic silica microparticles

Ferromagnetic microparticles were prepared according to Kilaas et al. (Kilaas, Dyrli, & Skagestad, 2004). Briefly, 20 g of ferromagnetic magnetite particles (size 200–300 nm) were dispersed in 40 g waterglass using an Ultraturrax (T25 digital, IKA, Staufen, Germany). An oil phase of toluene containing 30 g/L Span80 was added during the mixing. The resulting water-in-oil emulsion was stirred in a reactor at 20 °C and 30 mL 2 mol/L nitric acid was added. After stirring for 1 h and addition of 30 mL methanol, the suspension was stirred at 50 °C for 16 h. The final particles were washed with methanol and water. Note that a key factor when using ferromagnetic material for making "free" magnetic spheres will be the handling of the spheres during the manufacturing process. The coating, polymerization, washing and drying steps in the process should preferably be carried out without use of a magnetic device (to avoid premature introduction of remanence). If produced in this manner, the particles are freely dispersed (no agglomerates) until the application of a magnetic field.

2.2.3. Amine-functionalization of silica particles

2.2.3.1. Aminopropyl trimethoxysilane (ATMS-functionalized) particles.
Briefly, 20 g magnetic silica particles were dispersed in 400 mL toluene containing 40 mL aminopropyl trimethoxysilane (ATMS), and the mixture was bubbled with nitrogen for 15 min. Then the reaction was performed at 60 °C for 5 h. After the reaction, the particles were washed 5 times with methanol, and dried in an oven at 50 °C.

2.2.3.2. Jeffamine-functionalized particles. JEFFAMINE D-400 polyetheramine is characterized by repeating oxypropylene units in the backbone. As shown by the representative structure (Fig. 1), JEFFAMINE D-400 polyetheramine is a bifunctional, primary amine with average molecular weight of about 430 g/mol. The functionalization of the particles with Jeffamine was carried out in a two-step process. First, epoxy groups were introduced onto the surface of the magnetic silica particles by silanization with 3-glycidioxypropyl trimethoxysilane. Briefly, 20 g particles were dispersed in 400 mL toluene containing 56 mL 3-glycidyloxypropyl trimethoxysilane. The mixture was bubbled with nitrogen for 15 min. Then the reaction was performed at 60 °C for 5 h. After the reaction, the particles were washed 5 times with methanol, and dried at 50 °C. In the second step, 3 g of epoxy-coated silica particles were dispersed in 50 mL water, and then 12 mL of Jeffamine were added. The sample was placed in a water bath at 60 °C and incubated for 24 h. Afterwards the particles were washed 5 times with water.

2.2.3.3. Chitosan-functionalized particles. Ethanol was used to wet 1 g chitosan before the chitosan was dissolved in 60 mL water. The chitosan solution was added to 3 g of epoxy-coated silica particles (prepared as described above) under agitation, the sample was placed in a water bath at 60 °C and incubated for 24 h. Afterwards the particles were washed 5 times with water.

2.2.4. Enzyme immobilization methods

2.2.4.1. Glutaraldehyde activation of amine-functionalized silica particles. Typically, 200 mg particles were dispersed in 4 mL 0.25 mol/L glutaraldehyde in 0.1 mol/L pH 7.7 phosphate buffer and incubated for 4 h at 20 °C. Then the particles were washed 2 times in 10 mL 0.1 mol/L pH 7.7 phosphate buffer to remove excess glutaraldehyde, and finally dispersed in 4 mL 0.1 mol/L pH 7.7 phosphate buffer.

2.2.4.2. Covalent immobilization of protease to glutaraldehyde-activated particles. Immobilization was performed by dispersing 40 mg glutaraldehyde activated particles in 1 mL 0.1 mol/L pH 7.7 phosphate buffer containing protease, where the amount of protease was varied from 10 to 50 μg/mg particles. The incubation was performed for 18 h at 20 °C, and subsequently the samples were washed 3 times with 0.1 mol/L pH 7.7 phosphate buffer. Samples were mixed for 30 min between each wash. The particles were dispersed in 1 mL 0.1 mol/L pH 7.7 phosphate buffer and stored at 4 °C.

2.2.5. Hydrolysis of chicken meat and Turkey tendons

2.2.5.1. Single-run hydrolysis of chicken meat and Turkey tendons. **Sample preparation:** Suspensions of magnetic particles (with or without immobilized enzyme) at a concentration of 100 mg/mL were prepared in 0.01 mol/L pH 7.0 phosphate buffer and gently heated to 25 °C. The enzyme concentration was 50 μg/mg particles, resulting in immobilized amounts as given in Table 1 for each particle type. The total enzyme concentration for the hydrolysis experiments corresponds to ~0.05% per vial. One mL of the 100 mg/mL suspensions was added to Eppendorf tubes containing 200 mg of either chicken meat or turkey tendons. Negative and positive controls were 1 mL of 0.01 mol/L pH 7.0 phosphate buffer and 1 mL of 0.5 g/kg free protease in 0.01 mol/L pH 7.0 phosphate buffer, respectively. **Hydrolysis:** The samples were heated in a 45 °C water bath for 10 min, and then allowed to incubate at 42 °C for 3 h in a heating cabinet with gentle agitation. After the reaction was completed, the particles with meat or tendons were removed by magnetic separation, and the remaining solutions were transferred to new Eppendorf tubes and placed in a water bath at 95 °C for 20 min. After thermal deactivation, the samples were stored at −20 °C prior to analysis. The magnetic particles with meat or tendons were resuspended in 1 mL 0.01 mol/L pH 7.0 phosphate buffer and stored at 4 °C.

2.2.5.2. Repeated hydrolysis of chicken meat and Turkey tendons. Following the initial 3-h reaction, the remaining particles and meat (or tendons) were collected, redispersed in 1 mL 0.01 mol/L pH 7.0 phosphate buffer and stored at 4 °C overnight. Then the hydrolysis was carried out as described above. This procedure was repeated over 3 consecutive days, yielding a total reaction time of 12 h for each sample.

2.3. Characterization

Particle size measurements were performed using a Beckman Coulter

Table 1
Summary of key results for immobilized protease onto magnetic particles with three different coatings. ATMS = aminopropyl trimethoxysilane. Pooled variance for the relevant parameters was calculated based on triplicate measurements (analytical replicates) for each datapoint.

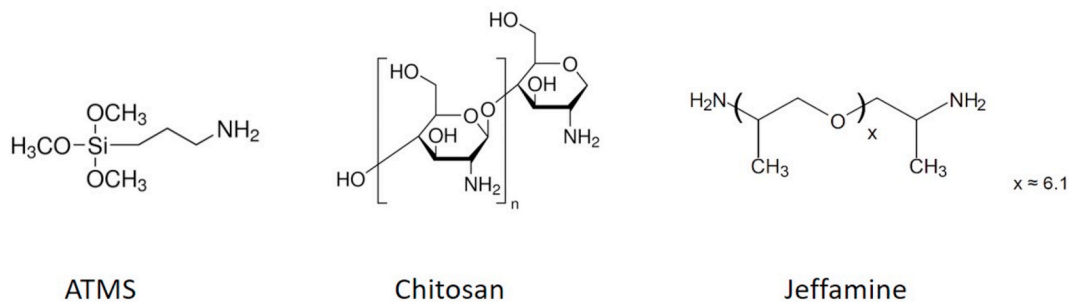| Particle coating	μmol amine/mg particles	Enzyme added μg/mg particles	Enzyme bound		
			μg/mg particles	% of added	μg/μmol surface amino groups
ATMS		10	5.1	50.6	10.9
	0.47	20	9.6	47.8	20.6
		40	19.9	49.9	42.9
		50	25.8	51.5	55.4
Chitosan	**0.19**	10	2.7	26.7	14.0
		20	5.7	28.6	30.1
		40	12.0	30.0	63.2
		50	14.7	29.4	77.4
Jeffamine	**0.05**	10	6.7	67.2	134.3
		20	8.2	41.0	164.0
		40	11.0	27.4	219.4
		50	11.8	23.6	235.7
Pooled variance	3×10^{-4}	N/A	0.97		

Fig. 1. Chemical structure of the three different coatings used on magnetic particles to facilitate immobilization of enzymes on the particle surface. ATMS = aminopropyl trimethoxysilane.

LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Brea, California, USA) with water as the mobile phase. The mean particle size of the base particles was 2.1 ± 0.8 μm, with a Sauter mean diameter ($D_{3,2}$) of 1.8 μm. The BET surface area of the base particles was determined via nitrogen adsorption (Micromeritics TriStar 3000 (Micromeritics, Norcross, Georgia, USA)) to be 25.7 ± 0.1 m^2/g.

2.3.1. Quantification of the number of amino groups on the particle surface

The number of terminal amino groups was estimated using the ninhydrin test. Briefly, ninhydrin was dissolved in DMSO together with SnCl$_2$ and diluted with lithium acetate buffer (pH 5.2). 500 μl ninhydrin solution was added to 0.5–4 mg particles and placed in a boiling water bath for 15 min. The samples were then cooled on ice for 15 min, and 2.5 mL of 480 g/L ethanol was added. The particles were removed with a magnet, and aliquots of the supernatant were extracted and their absorbance at 570 nm were measured on a plate reader (BioTek Synergy H1 hybrid multi-mode microplate reader, BioTek, Winooski, Vermont, USA).

2.3.2. Quantification of the amount of protease immobilized

The amount of protease immobilized to the magnetic particles was measured indirectly by measuring the amount of protease in the supernatant and in the washing buffers, followed by subtraction of the measured values from the amount of protease added. The amount of protease was measured using a modified Bradford method (Bradford, 1976; Ernst & Zor, 2010). The absorbance of the samples was measured at 450 nm and 590 nm using a plate reader (BioTek Synergy H1 hybrid multi-mode microplate reader), where the ratio of the absorbance values at 590 nm and 450 nm is first-order linear with respect to protein concentration.

2.3.3. Activity of immobilized protease

The activity of the immobilized enzymes was estimated using azo-casein. The magnetic particles with immobilized enzymes were washed once in 0.1 mol/L pH 7.0 phosphate buffer, 2 mg particles were dispersed in 100 μL phosphate buffer and 100 μL solution of 10 g/L azo-casein was added. The mixture was incubated for 60 min at 40 °C before the particles were separated with a magnet and the supernatant removed. Then 200 μL 110 mmol/L trichloroacetic acid was added to the supernatant in order to precipitate the remaining azo-casein. The supernatant was removed and the absorbance at 440 nm was measured using a plate reader (BioTek Synergy H1 hybrid multi-mode microplate reader).

2.3.4. Size exclusion chromatography

Protein hydrolysates of chicken meat and turkey tendons were analyzed with size exclusion chromatography (SEC) using a separation method described by Wubshet et al. (Wubshet et al., 2017). In brief, the SEC analyses were performed using either an Agilent 1200 series instrument (Santa Clara, California, USA) or a Dionex Ultimate 3000 series instrument (Thermo Scientific, Waltham, Massachusetts, USA) equipped with a quaternary pump, an autosampler and a UV–Vis detector. The water phase from the EPH were directly used as injection solutions (10 μL) and chromatographic separation was performed at 25 °C using a BioSep-SEC-s2000 column (300 × 7.8 mm, Phenomenex, Torrence, California, USA). The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion 30:70 (v/v), containing 0.05 ml/L trifluoroacetic acid. Isocratic elution was carried out using a flow rate of 0.9 mL/min for 20.0 min. Between 20.0 and 20.1 min the mobile phase was changed to NaH$_2$PO$_4$ (0.10 mol/L) and maintained until 23.0 min for column cleaning. Elution conditions were restored between minute 23.0 and 23.1 and the column was equilibrated for an additional 27 min. Chromatographic runs were controlled from either OpenLAB CDS Rev. C.01.07 (Agilent Technologies, Inc., Santa Clara, California, USA) or Chromeleon™ Chromatography Data System (CDS) software (Thermo Fisher Scientific). Using molecular weight standards, average molecular

weight of the peptides and proteins in the hydrolysates was calculated from the UV chromatograms (214 nm) as described in Wubshet et al., 2017. The total area under the SEC trace (A) and average molecular weight (M_w) were calculated using PSS winGPC UniChrom V 8.33 (Polymer Standards Service, Mainz, Germany) for each chromatogram.

2.4. Statistical analysis

All measurements were performed in triplicate (analytical replicates). Microsoft Excel was used for calculating average and standard deviation. Curve fitting of linearized adsorption isotherms was done using GraphPad Prism version 9.1.0.

3. Results and discussion

3.1. The coupling strategy used determines the number and density of binding sites

The magnetic silica particles were coated using three different coupling strategies to investigate the effect of the surface chemistry and topography on the enzymatic activity of the immobilized enzymes. A summary of the amounts of amino groups introduced onto the particles with the tree different coating methods is shown in Table 1 (left column).

In the first case, the particles were silanized with aminopropyl trimethoxysilane (ATMS – see Fig. 1 for chemical structure), resulting in a dense layer with a high density of amino groups with the potential to bind high amounts of enzyme. As expected, coating the magnetic particles with the low molecular weight linker ATMS resulted in the highest surface concentration of amino groups of the coatings used here.

In the second case, epoxy silica particles were coated with chitosan resulting in a hydrophilic gel-like coating with amino groups, which may be suitable for the protease activity of the immobilized enzymes. Since chitosan has only one amino group per unit, and the hydrogel-like chitosan surface layer is much less dense than for ATMS, the lower observed surface concentration of amino groups is not unreasonable.

In the third case, JEFFAMINE D-400 polyetheramine (hereafter referred to as Jeffamine) was coupled to epoxy-functionalized silica particles via the same two-step process as for chitosan. The surface concentration of amino groups was significantly lower for the Jeffamine-coated particles compared to the other coatings (~10x lower as compared to ATMS and ~4x lower than for chitosan). This could be attributed to at least three factors – either alone or in combination: (i) a reduced number of binding sites compared to the ATMS coating due to the epoxidation step, (ii) submonolayer coverage owing to steric hindrance from bound Jeffamine molecules, or (iii) reaction between surface epoxy groups and both amines for a fraction of the Jeffamine molecules, thus leading to both a reduced surface coverage and a lower concentration of unreacted amino groups.

Thus, the three coating strategies can be ranked in terms of available amino groups for functionalization as: ATMS > chitosan > Jeffamine.

3.2. The particle coating dictates amount and binding efficiency of the immobilized protease

Table 1 and Fig. 2 show the protease immobilized to the three glutaraldehyde-activated particles as a function of the amount of protease added, confirming that protease was immobilized onto all three particle coatings. Consequently, all three coupling strategies were used as a template for protease immobilization.

Particles coated with ATMS bind more protease than the other two coatings, which could be expected since these particles have a higher amount of amino groups on the surface compared the other coatings (Fig. 2a and Table 1). None of the coatings display a typical high-affinity binding isotherm, *i.e.;* rapid initial binding followed by a saturation plateau, for the concentration range used here. Instead, enzyme
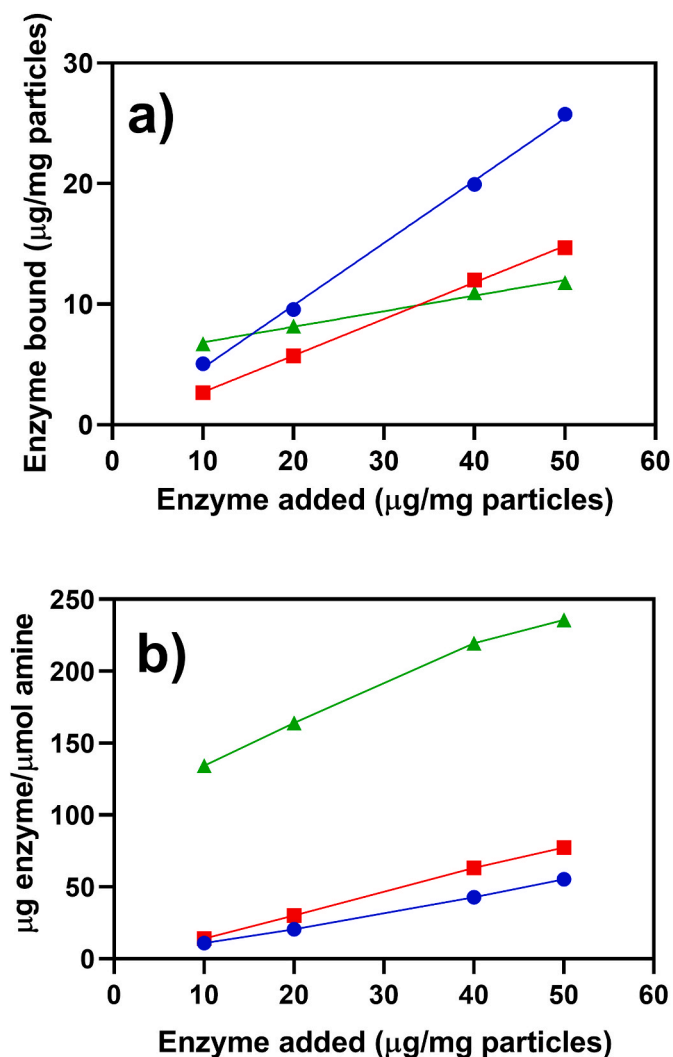
Fig. 2. Protease immobilized on particles as a function of amount of protease added for the three different particle coatings used in terms of a) the amount of immobilized enzyme per mass unit particle, and b) in terms of immobilized amount of enzyme relative to the surface concentration of amine groups. ● ATMS (aminopropyl trimethoxysilane), ■ Chitosan and ▲ Jeffamine coating.

Table 2

Adsorption isotherm parameters for the immobilization of protease onto glutaraldehyde-activated aminopropyl trimethoxysilane (ATMS), Chitosan and Jeffamine-coated magnetic particles.

Isotherm model	Parameters	ATMS	Chitosan	Jeffamine
Langmuir	Goodness of Fit	$R^2 = 0.186$	$R^2 = 0.739$	$R^2 = 0.991$
	Suited/not suited	Not suited	Not suited	Suited
	q_{mono} (µg/mg particles)	–	–	14.9
	K_L	–	–	0.07
	R_L	–	–	0.02–0.09
Freundlich	Goodness of Fit	$R^2 = 0.998$	$R^2 = 0.884$	$R^2 = 0.998$
	Suited/not suited	Suited	Partly suited	Suited
	K_F	0.48	4.32	0.35
	$1/n$	1.01	~0	0.64
Temkin	Goodness of Fit	$R^2 = 0.062$	$R^2 = 0.883$	$R^2 = 0.989$
	Suited/not suited	Not suited	Partly suited	Suited
	RT/b_T	–	~0	1.68
	K_T	–	~0	0.23
Hill-Langmuir	Goodness of Fit	N/A	N/A	$R^2 = 0.930$
	Suited/not suited	Not suited	Not suited	Suited
	n_H	–	–	0.62

could be due to geometric effects such as surface roughness/particle porosity in the case of ATMS-coated particles, or network restraints in the case of the chitosan hydrogel shell layer.

Both the ATMS and Jeffamine coatings were found to be well suited for the Freundlich model, with correlation coefficients close to unity (Table 2). For the chitosan coating, the Freundlich isotherm model yields a moderately strong correlation between the variables, and is thus denoted "partly suitable," likely owing to too few data points. A good fit to the Freundlich isotherm generally suggests that the surface is heterogeneous, which is in agreement with the surface area measurements of the base particles, which indicate surface roughness and/or some porosity. This also reflected in the Freundlich fit parameters for the ATMS and Jeffamine coatings, where shortest linker (ATMS) yields a higher adsorption capacity (K_F) and a higher distribution of surface energies ($1/n$) compared to Jeffamine. These fit parameters are largely in agreement with experimental observations (Table 1), where the ATMS coating resulted in the highest amount of immobilized enzyme per particle. It is also reasonable that the particles with the shorter linker (ATMS) are more susceptible to surface roughness effects than the longer Jeffamine linker. While the poorer fit for chitosan does not allow for quantitative comparison to the two other coatings studied here, the Freundlich model qualitatively fits the system, which is in agreement with earlier publications on adsorption of lipase onto chitosan (Gilani et al., 2016a; Gilani, Najafpour, Moghadamnia, & Kamaruddin, 2016b) as well as other commercial supports (Gitlesen et al., 1997).

The Temkin adsorption isotherm model has previously been reported to fit glutaraldehyde-activated immobilization of lipase onto chitosan (Gilani et al., 2016a, 2016b). From the adsorption isotherm fitting parameters (Table 2), the Temkin isotherm is well suited to the Jeffamine system and partly suited to the chitosan system, confirming chemical adsorption. Interestingly, the Temkin isotherm provided a very poor fit to the ATMS coating, which may be indicative of a very heterogeneous surface for the ATMS-coating as discussed above.

From the fitting of the immobilized enzyme to various adsorption isotherm models, it can be concluded that the base particle surface is heterogeneous, resulting in a distribution of adsorption energies as opposed to a uniform surface with equivalent adsorption sites. In turn, this results in different binding affinities for the three coatings used here.

immobilization appears to follow an approximately first-order linear relationship with respect to the bulk concentration of enzyme. In order to further investigate the immobilization characteristics, four common adsorption isotherms were applied to the data set: the Langmuir isotherm, the Freundlich isotherm, the Temkin isotherm and the Hill-Langmuir isotherm, see supplementary information for a description of each adsorption isotherm.

The Langmuir adsorption isotherm has been reported to describe enzyme adsorption well (Al-Duri & Yong, 2000; Gitlesen, Bauer, & Adlercreutz, 1997) – including binding of lipase onto glutaraldehyde-activated chitosan supports (Gilani, Najafpour, Moghadamnia, & Kamaruddin, 2016a). Here, only the Jeffamine-coated particles showed a good fit to the Langmuir model (Table 2), with the corresponding R_L values indicating that immobilization of protease onto glutaraldehyde-activated Jeffamine beads is favorable and irreversible. Complete monolayer coverage for the Jeffamine particles was estimated to be 14.9 µg enzyme per mg particles. From the Hill-Langmuir model, Jeffamine coatings were found to be anti-cooperative, *i.e.;* the binding affinity decreases with fractional surface coverage (Table 2). The other two coatings – particularly ATMS-coated particles – showed a poor fit to the Langmuir isotherm model within this concentration range. This

3.3. Activity of immobilized protease is inversely correlated to surface coverage

The immobilized enzymes were active, and the total activity in katals (pkat/mg particles) did not differ significantly between the three coatings within the enzyme concentration range studied (Table 3). For all three coatings, the activity of immobilized enzyme was lower than for the free enzyme. This observation is quite common; in a 2007 review, Sheldon (Sheldon, 2007) reported that immobilization often leads to loss of more than 50% of activity compared to the free/native enzyme, particularly at higher surface coverage. The lower activity of immobilized enzymes could indicate that the immobilized enzymes are sterically hindered, either by surface crowding or due to immobilization also occurring within pores or on surface regions with higher curvature/roughness. The latter hypothesis is supported by the observation that chitosan-coated particles show the highest initial activity (pkat/μg enzyme) relative to free enzyme. Specifically, immobilization onto the gel-like chitosan coating and subsequent enzyme activity is much less likely to be affected by particle porosity or surface roughness compared to the smaller Jeffamine or – especially – ATMS coatings. Further, the total activity (pkat/mg particles) did not increase significantly with increasing amounts of immobilized enzyme, which is in agreement with reported literature (see e.g. review by Sheldon (Sheldon, 2007) and references therein), and likely also supports the steric hindrance hypothesis (Table 3). As seen in Fig. 2, the amount of enzyme immobilized increased with increasing amount of enzyme added, however, the higher amount of immobilized enzyme does not lead to higher activity. This is illustrated in Fig. 3 where the enzyme activity is presented as a function of the amount of enzyme added in the immobilization step. When more enzyme is packed on the surface, it may not be available for the substrate, and in addition enzymes may not be able to rotate freely, hence the activity may be reduced. A similar observation has been reported e.g. for immobilization of lipase, where enzyme activity was found to stabilize or decline beyond a certain loading (Al-Duri & Yong, 2000). In the case of Jeffamine-coated particles, the flexible chains may increase the mobility of the immobilized enzymes thereby increasing the protease activity.

An alternative explanation for the observed decrease in activity with higher loading could be proteolysis, whereby neighboring enzymes degrade each other. However, in their 2011 review, Garcia-Galan et al. (Garcia-Galan et al., 2011) specifically mention immobilization as a mitigation strategy for proteolysis. The flexibility, amount and dimensionality of immobilized enzymes should also be taken into account, as these parameters dictate whether neighboring enzymes are sufficiently close to interact. For the systems studied here, the ATMS coating has the highest amount of immobilized enzymes both with respect to absolute amount and dimensionality (i.e.; distributed onto the base particle surface and not in a 3D gel as for chitosan). Consequently, proteolysis should be most prominent for the ATMS-coated particles. However, this is not the case, as seen from the activity and trends in Table 3 and Fig. 3 (a and b panels). Thus, while we cannot rule out deactivation by proteolysis, we do not see any robust trends in our data to support this hypothesis.

Identification of the optimum amounts of immobilized enzyme is of high practical importance as it indicates that it is not necessary to add
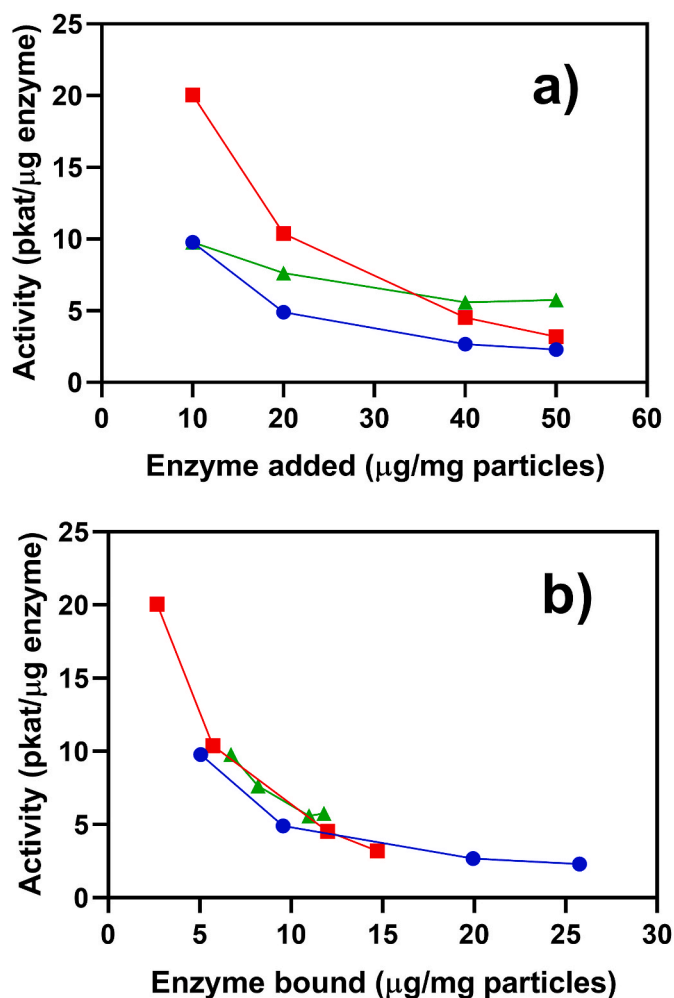

Fig. 3. Activity of immobilized enzyme (pkat/μg enzyme) on magnetic particles with different coatings as a function of a) added, and b) particle-bound enzyme. ● ATMS (aminopropyl trimethoxysilane), ■ Chitosan and ▲ Jeffamine coating.

high amounts of costly enzymes to obtain a particle with good activity, as also reported by Al-Duri and Yong (Al-Duri & Yong, 2000). Moreover, for larger substrates it might be beneficial to immobilize enzymes onto a smooth particle surface in order to facilitate contact between enzyme and substrate.

3.4. Activity of immobilized enzyme is retained over repeated measurements and after storage

Operational stability– i.e.; recycling, reuse and storage stability - remains one of the most important goals for immobilized enzyme constructs. To investigate these crucial parameters, we have studied recyclability of the immobilized enzyme over six cycles, as well as enzymatic activity after storage over 25 months.

Table 3

Activity of free and immobilized enzyme in picokatals (pkat) on the three different coated particles studied here. ATMS = aminopropyl trimethoxysilane. The pooled variance for activity for immobilized enzyme based on triplicate measurements (analytical replicates) was calculated to be 0.57 pkat/μg enzyme.

Enzyme added	Free enzyme	ATMS-particles		Chitosan-particles		Jeffamine-particles	
μg/mg particles	pkat/μg enzyme	pkat/mg particles	pkat/μg enzyme	pkat/mg particles	pkat/μg enzyme	pkat/mg particles	pkat/μg enzyme
10	23.0	49.5	9.8	53.5	20.1	65.5	9.8
20	–	46.8	4.9	59.4	10.4	62.5	7.6
40	–	53.4	2.7	54.3	4.5	61.4	5.6
50	–	59.5	2.3	46.9	3.2	68.1	5.8

The stability of the immobilized enzyme was investigated by repeating the activity measurements 6 times over a period of two weeks. After each activity measurement the particles were washed and stored at 4 °C until the next experiment. As shown in Fig. 4, the activity was relatively stable during this period, with immobilized enzyme retaining approximately 85%, 75% and 65% of their initial activity for ATMS, chitosan and Jeffamine coatings, respectively. Both the observed trend and level of enzyme activity over several cycles are in line with what has been reported for trypsin (Aslani et al., 2018) and lipase (Ranjbakhsh, Bordbar, Abbasi, Khosropour, & Shams, 2012) immobilized onto silica-coated magnetite nanoparticles, and for protease immobilized onto mesoporous silica nanospheres (Ibrahim et al., 2016). While the present dataset does not allow for identification of the cause of the activity loss over repeated measurements, likely causes include interaction with hydrophobic interfaces such as air/gas bubbles and EPH byproducts, or proteolysis from tight packing of neighboring proteases. Changes in local pH over time as a function of base particle degradation has also been proposed as an inactivation mechanism in the case of magnetic metal-organic frameworks (MOFs) (Feng et al., 2021). However, the hydrolytic stabilities of MOFs and the magnetic silica particles used here are expected to differ significantly, owing both to composition and porosity. For a review of inactivation routes of immobilized enzymes, see e.g. Garcia-Galan (Garcia-Galan et al., 2011) and references therein.

In order to investigate storage stability, the particles with immobilized enzyme were stored at 4 °C, and enzyme activity was measured after 6 months, 16 months and 25 months storage. After storage for half a year no loss in activity was observed, whereas after 25 months storage, the activity was retained at 83%, 79% and 93% of the initial activity for ATMS, chitosan and Jeffamine, respectively. Thus, the immobilized enzymes studies here show good operational stability with respect to industrial use.

3.5. Hydrolysis of chicken meat and turkey tendons

In order to further evaluate the applicability of the developed immobilized enzyme on the three different particle types, a proof-of-concept study was performed where the particles were used to catalyze hydrolysis of chicken meat and turkey tendons. The two raw materials were chosen to provide two test systems ranging from easy to digest muscle myofibril protein (*i.e.;* chicken muscle) to hard to hydrolyze connective tissue (*i.e.;* turkey tendon). A summary of results showing average molecular weight and total chromatographic area from

size exclusion chromatography analysis of protein hydrolysates produced using the immobilized enzymes is presented in Table 4. Generally, compared to the hydrolysis controls (both buffer and enzyme-free particle control), hydrolysates from all the three particle-immobilized enzyme systems were characterized by lower molecular weight (*i.e.;* increased degree of hydrolysis) and higher total area (*i.e.;* increased protein yield). This indicates that all the three particle-immobilized enzyme systems were able to catalyze degradation and extraction of protein form chicken meat and turkey tendons. No significant difference was observed between the activity of the three immobilized enzyme systems and all were less active than the free protease which resulted in highest protein yield (*i.e.;* highest total area). One interesting observation was the difference in the hydrolysates resulting from the enzyme-free control particles. In both the tendon and meat hydrolysis, the Jeffamine control particles, characterized by the enzyme being anchored to flexible chains, resulted in lower molecular weight peptides. This could potentially be a result of better substrate availability owing to the higher enzyme mobility as discussed above.

One important aspect of immobilizing industrially relevant enzymes from protein hydrolysis is reusability (Aslani et al., 2018; Ibrahim et al., 2016; Ranjbakhsh et al., 2012). In order to evaluate reusability of the immobilized enzymes, a total of 4 cycles of hydrolysis reactions was performed using the same particles. The results (average molecular weight and total chromatographic area) from the immobilized enzymes together with the control reactions using unfunctionalized particles are presented in Figs. 5–8. No significant difference could be observed in the molecular weight of the resulting peptides from the four cycles of hydrolysis reactions. However, an apparent decrease in protein yield (as estimated from the total areas, Fig. 6) was observed from the first to the fourth cycle. This significant exponential decrease is mainly attributed to loss of particles in each cycles of reactions and not due to loss of activity. This is evident from the observation even with the control particles (*i.e.;* without immobilized enzyme), as well as from the repeated protease activity studies presented above. The control enzyme-free particles likely contribute to increasing protein extraction and hence protein yield by mechanical interactions, *i.e.;* grinding during
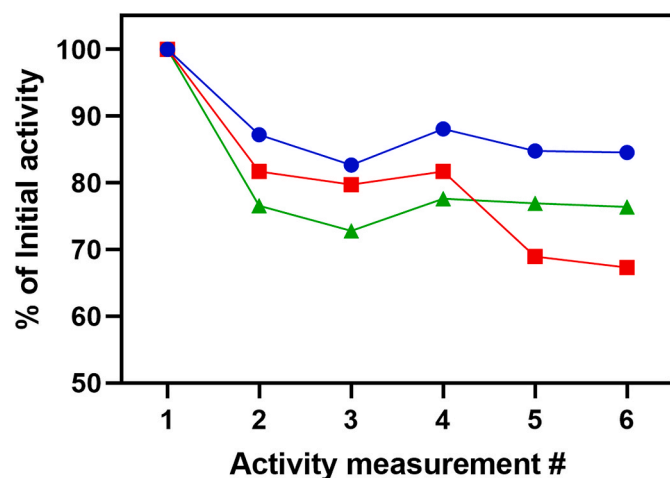
Fig. 4. Reusability of enzyme immobilized onto magnetic particles with three different coatings as measured by repeating the activity measurement 6 times of each particle type over a period of two weeks. ● ATMS (aminopropyl trimethoxysilane), ■ Chitosan and ▲ Jeffamine coating.

Table 4
Summary of molecular weight and total chromatographic area from size exclusion chromatography of hydrolysis products when performing hydrolysis reaction on chicken meat and turkey tendons, using enzyme immobilized on magnetic particles with three different coatings. Hydrolysis results for the particles without enzymes are shown as controls, together with free protease and buffer only. ATMS = aminopropyl trimethoxysilane. A.U = Arbitrary Units.

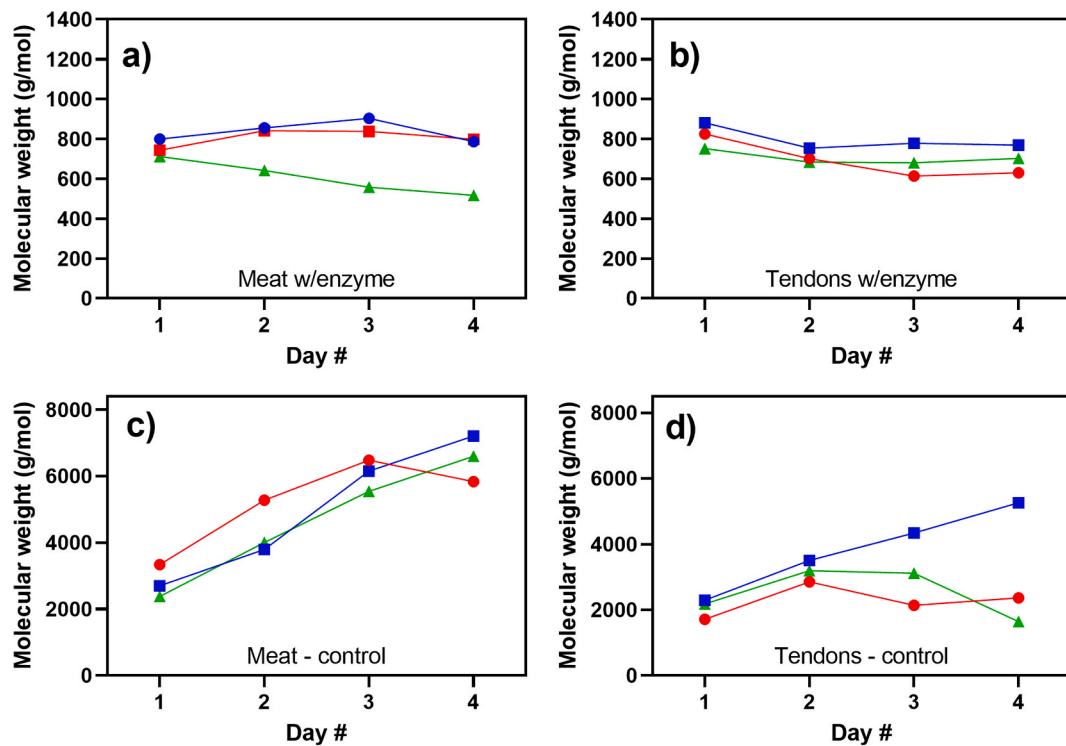|	Chicken meat		Turkey tendons	
	Average molecular weight (g/mol)	Total Area (A. U)	Average molecular weight (g/mol)	Total Area (A. U)
Protease only	742	105	4752	125
ATMS-Protease	860	43	1090	30
ATMS-Protease, stored	979	56	1033	25
Chitosan-protease	934	39	1087	25
Chitosan-Protease, stored	905	38	876	20
Jeffamine-Protease	798	54	880	25
Jeffamine-Protease, stored	895	61	943	22
ATMS, control	3077	17	2145	16
Chitosan, control	1904	22	2027	17
Jeffamine, control	1831	21	1920	15
Buffer only	5775	26	–	0.1

Fig. 5. Molecular weights of hydrolysis products when performing four cycles of hydrolysis reaction on chicken meat and turkey tendons, using enzyme immobilized on magnetic particles with three different coatings. Figure a) hydrolysis of chicken meat using immobilized enzymes, b) hydrolysis of turkey tendons using immobilized enzymes, c) hydrolysis of chicken meat using magnetic particles without enzymes as control, d) hydrolysis of turkey tendons using magnetic particles without enzymes as control. ● ATMS (aminopropyl trimethoxysilane), ■ Chitosan and ▲ Jeffamine coating.
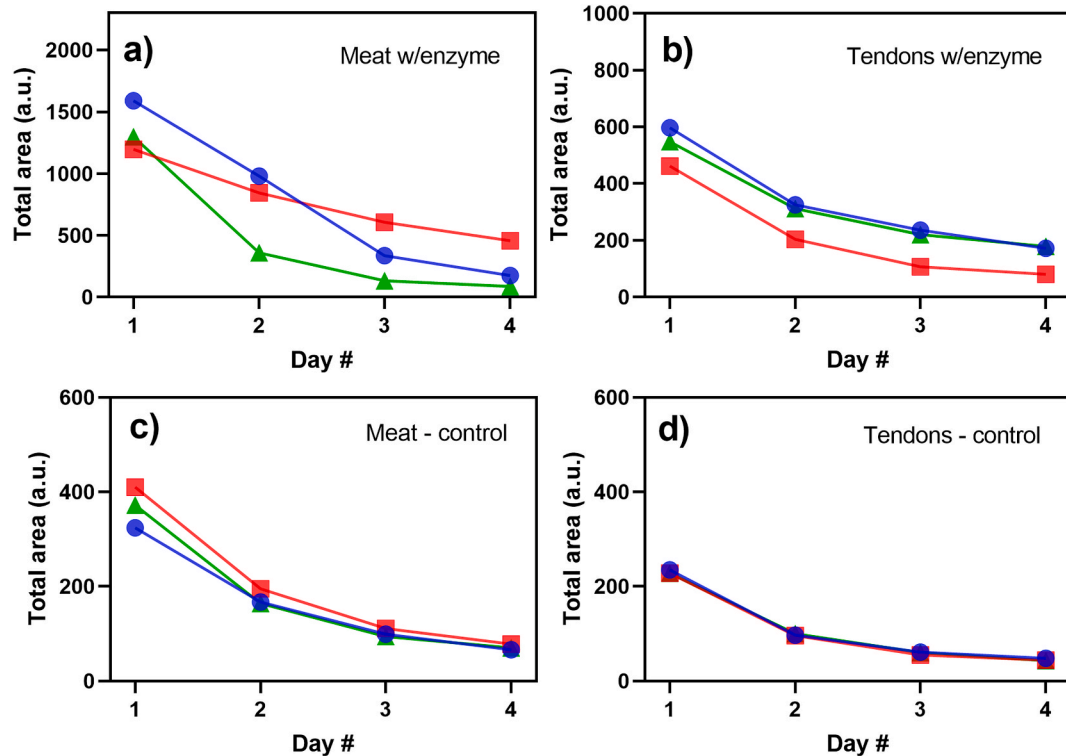

Fig. 6. Development in total chromatographic area from size exclusion chromatography of hydrolysis products when performing four cycles of hydrolysis reaction on chicken meat and turkey tendons, using enzyme immobilized on magnetic particles with three different coatings. Figure a) hydrolysis of chicken meat using immobilized enzymes, b) hydrolysis of turkey tendons using immobilized enzymes, c) hydrolysis of chicken meat using magnetic particles without enzymes as control, d) hydrolysis of turkey tendons using magnetic particles without enzymes as control. ● ATMS (aminopropyl trimethoxysilane), ■ Chitosan and ▲ Jeffamine coating.
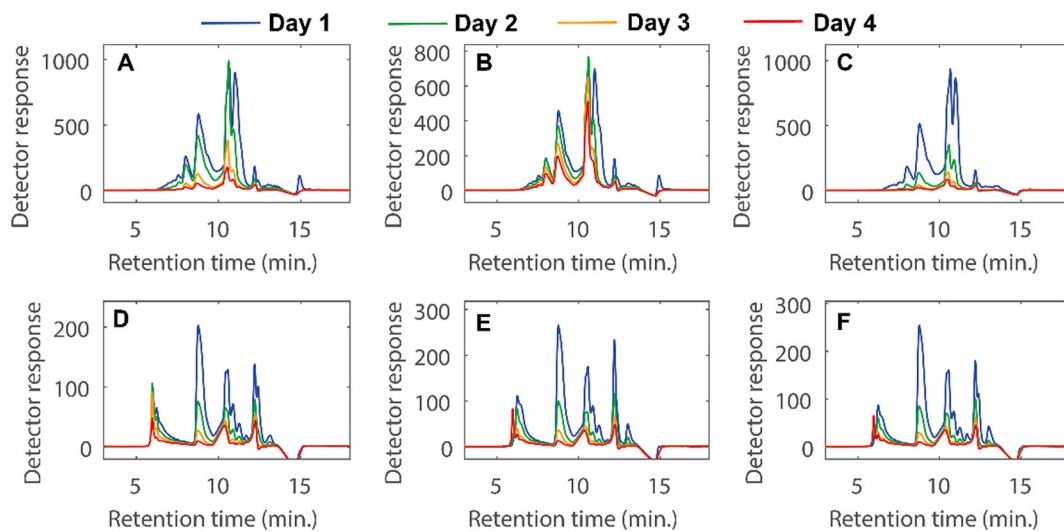
Fig. 7. Size exclusion chromatographic traces of hydrolysis products from chicken meat hydrolysis when performing four cycles of hydrolysis reaction, using aminopropyl trimethoxysilane (ATMS) beads with (A) and without (D) enzyme, chitosan beads with (B) and without (E) enzyme, and jeffamine beads with (C) and without (F) enzyme.
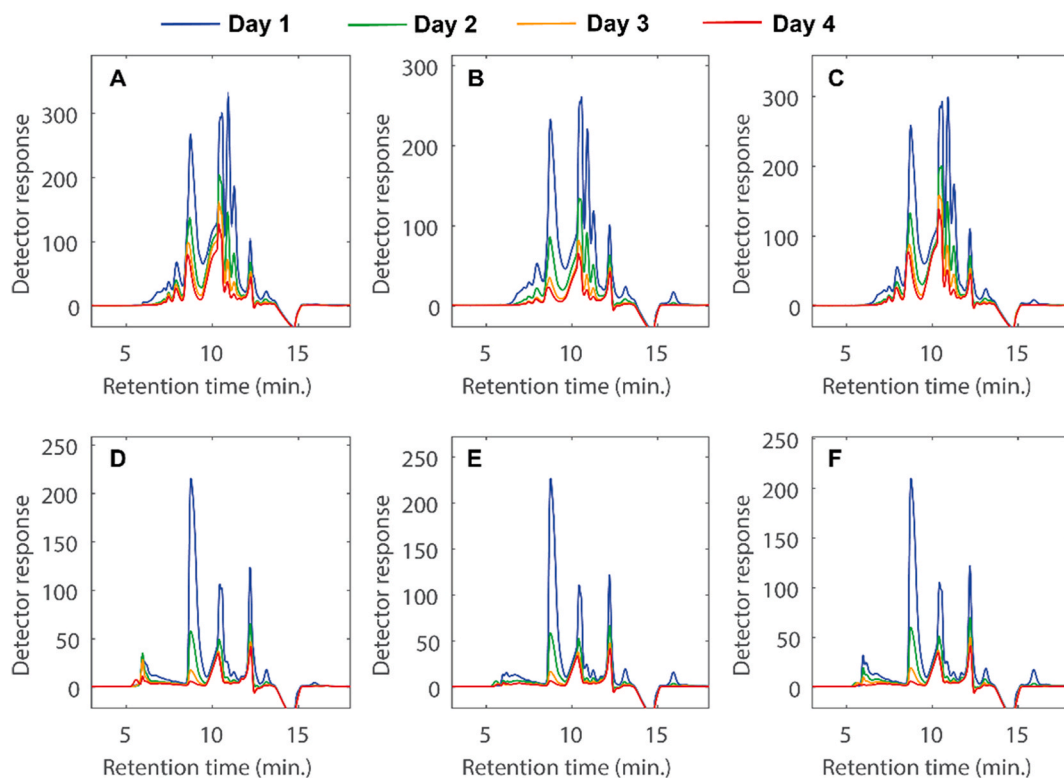

Fig. 8. Size exclusion chromatographic traces of hydrolysis products from turkey tendon hydrolysis when performing four cycles of hydrolysis reaction, using aminopropyl trimethoxysilane (ATMS) beads with (A) and without (D) enzyme, chitosan beads with (B) and without (E) enzyme, and jeffamine beads with (C) and without (F) enzyme.

hydrolysis. Since the enzyme-free particles also show a similar trend in decreased protein yield, it indicates that poor particle recovery plays a significant role in decrease in yield during the repeated use. This is not surprising taking into account the inherent chemical and physical complexities of the raw materials used in the current study. Thus, a better particle recovery strategy is expected to boost the reusability of the immobilized enzymes.

4. Conclusions

The protease Subtilisin A was covalently immobilized onto magnetic silica particles using three different amine ligands; a short, brush-like linker (ATMS), a long, flexible linker (Jeffamine), and a gel-like coating (chitosan). Particles coated with ATMS were found to have the highest amount of amino groups on the surface, and consequently was found to have the highest protease loading of the systems studied. The immobilized enzymes showed similar total activity (pkat/mg particles)

for all three coatings investigated here. Moreover, the enzyme activity was retained after six consecutive cycles and after 16 months storage, indicating good operational stability. Thus, a robust and scalable covalent coupling of enzymes onto magnetic silica particles can be obtained, which can facilitate the use in industrial EPH of by-products. A drawback is a lower activity level compared to the free enzyme. Total activity was not found to scale with the amount of immobilized enzyme, indicating that maximum activity can be obtained at a rather low surface coverage. All the three particle-immobilized enzyme systems were able to catalyze degradation and extraction of proteins from complex model reaction mixtures comprising either chicken meat or turkey tendons as opposed to from simple protein solutions. Moreover, the immobilized systems could be recycled and reused after storage. Some additional effects were observed for the enzyme-free controls, likely owing to mechanical degradation resulting from the magnetic silica particles. The results reported here indicate that it is feasible to recover and reuse magnetic silica particles from the highly complex reaction mixture used in enzymatic protein hydrolysis. The study represents one of the first examples of the use of immobilized protease to catalyze hydrolysis of complex and industrially relevant raw materials.

CRediT authorship contribution statement

Wilhelm R. Glomm: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Sileshi G. Wubshet:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Diana Lindberg:** Conceptualization, Writing – original draft. **Katinka R. Dankel:** Investigation, Writing – original draft, Writing – review & editing. **Nils K. Afseth:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Per M. Stenstad:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Supervision. **Heidi Johnsen:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112327.

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