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Synthesis of a novel chitosan-based Ce(IV) complex with proteolytic activity *in vitro* toward edible biological proteins

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ABSTRACT

The occurrence of enzymatic activities is attributed to proper spatial organization of functional groups from first principles. A novel chitosan-based Ce(IV) complex (CC[Ce(IV)]), an artificial metalloproteinase, was synthesized by attaching cyclen, Ce(IV), and chlorophyll–Cu(II) to a chitosan-based matrix. The enzymatic hydrolytic efficiency (HE) and the procedure of catalyzing myoglobin (Mb) by CC[Ce(IV)] *in vitro* were investigated using spectrophotometry, electrophoresis, and liquid chromatography. The results showed that the HE of Mb was up to 60% at 60 °C within 24 h, displaying a catalytic proficiency. The pseudo-first-order kinetic constant (k_{obs}) for CC[Ce(IV)] treatment within 24 h was 3.85×10^{-2} h^{-1}, higher than that for α-chymotrypsin treatment, which was 2.63×10^{-2} h^{-1}. Moreover, the peptide bond derived from Asp-Phe/Phe-Asp in Mb could be specifically cleaved by CC[Ce(IV)], which could simulate the functionality of α-chymotrypsin. This work provides an experimental basis for potential utilization of the chitosan-based Ce(IV) complexes in the food industry.

1. Introduction

Biological proteins are generally hydrolyzed *via* natural enzymatic proteolysis pathways or microbial fermentation in the food industry. Some bioactive and flavor peptide products fabricated *via* proteolysis have been applied as functional foods, such as natto sauce, soy sauce, fish sauce, fermented bean curd, and yogurt (Wang, Wang, Liang, & Liang, 2013). However, some disadvantages of natural proteinases cannot be neglected. These include inseparability in reaction systems, antigenicity (excretive enzymes or antigenic membrane proteins from microorganisms), and risk of deactivation. Accordingly, the design of alternative artificial proteinases is of great attraction for potential applications in food production.

As an important branch of molecular biomimetics, artificial enzymes have been widely used in chemistry, chemical engineering, materials science, biology, and other fields *via* utilizing methods of analogy, simulation, and modeling to study abiological and biological enzymatic catalysis (Song & Tezcan, 2014). Currently, artificial metalloproteases have received the most attention

Abbreviations: DD, deacetylation degree; HAc, acetic acid; DMF, *N,N*-dimethylformamide; CC, chitosan–cyclen; CC[Ce(IV)], chitosan–cyclen chelating Ce(IV); cyclen, 1,4,7,10-tetrazacyclododecane; Mb, myoglobin; HE, hydrolytic efficiency; LC, liquid chromatography; SEM, scanning electron microscope; OM, optical microscope; LPSA, laser particle size analyzer; FTIR, Fourier transform infrared spectra; ICP-AES, inductively coupled plasma atomic emission spectrometry; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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for their structural similarity to "catalytic center" found in natural enzymes. In terms of natural enzymes, Mg(II), Zn(II), Ca(II), and Mn(II) are generally located in active center (Tyndall, Nall, & Fairlie, 2005). Likewise, transitional elements, e.g. Cu(II), Co(III), Fe(II), and Ni(II), have been widely applied to enzyme model designs (Shrivastava, Kanthimathi, & Nair, 2002; Kajimura, Sumaoka, & Komiyama, 1998). Rare earth elements, e.g. La(III), Ce(III), and Ce(IV), can form stable metal complexes with crown ether and macrocyclic polyamines because of their strong Lewis acidity, high coordination number, and fast ligand exchange speed. Thus, these metal ions and their complexes have been considered for protein and polypeptide cleavage (Xu & Qu, 2014).

Macrocyclic polyamines are a series of specific substances with a cavity structure. As a typical representative in the macrocyclic polyamine family, cyclen and its derivatives exhibit minimal steric hindrance and high metal selectivity to maximize their functions. Consequently, cyclen is commonly applied to chemical hydrolysis and radioactive diagnosis (Kassai & Grant, 2008). It has been reported that macrocyclic compounds conjugating with some metals are capable of cleaving peptide bonds *via* a hydrolytic pathway (Kim, Jang, Cheon, Suh, & Suh, 2009).

Chitosan, an abundant and natural polysaccharide derived from the shells of shrimps and crabs, is easily chemically modified because of the presence of functional groups such as hydroxyl, amine, and acetamido groups, and is also an ideal material of macromolecular biopolymers that serve as carriers (Roosen, Spooren, & Binnemans, 2014). Considering the construction of artificial enzymes, the support tethering functional groups such as cyclodextrin, crown ether, cyclen, and porphyrin not only can enhance the heat stability of chitosan-based matrices, but can also improve the recognition of protein substrates (Wang, Ding, Liu, & Wang, 2015). These functional groups are grafted onto the surface of a spherical matrix, thereby meeting the functionality of catalytic microdomains in view of nature employing peptides with various side chains as a skeleton of enzymes (Fan et al., 2014). Moreover, the insoluble matrix can similarly facilitate the separation between synthetic enzymes and products.

In nature, enzymes often retain specific structures as well as their functionalities. Their selective proteolysis in biocatalytic pathways underscore the peculiarity of such reactions. Varying the composition or three-dimensional architectures of the catalytic center can directly affect the catalytic features and substrate selectivities (Song & Tezcan, 2014; Yoo, Lee, Kim, & Suh, 2005). The above-mentioned relationship normally provides inspirations for the design of synthetic enzymes. Since the 1960s, numerous reports on artificial metalloproteinases have paid attention to the potential affinities between small-molecule catalysts and small-molecule substrates (Raynal, Ballester, Vidal-Ferran, & Van Leeuwen, 2014a,b; Motherwell, Bingham, & Six, 2001). Therefore, in this work we attempted to link cyclen, Ce(IV), and chlorophyll–Cu(II) to the skeleton of a chitosan-based matrix, which was degradable and environmentally friendly in the food and drug industry (Justin & Chen, 2014). We designed an insoluble proteolytic macromolecular catalyst with minimal interference affected by strong solvent polarity. For adherence to functional requirements, α-chymotrypsin from bovine pancreas, a typical endopeptidase, has been considered as a control in this study to assess the effect of limited proteolysis by the synthetic catalyst. Subsequently, the hydrolytic efficiency and possible cleavage mechanism of typical edible macromolecular myoglobin (Mb) catalyzed *in vitro* were investigated by spectrophotometry, electrophoresis, and liquid chromatography (LC). We aimed to seek a viable alternative to sensitive natural proteases. Likewise, the catalytic properties for other edible proteins such as ovalbumin and wine proteins were measured to evaluate the potential practical applications in the food industry.

2. Materials and methods

2.1. Materials

Chitosan powder (*M.W.* 550 k, deacetylation degree 92.3%, viscosity 1200 mPa s) was provided by Hecreat Marine Bio-Tech. Co., Ltd. (Qingdao, China) and used without further purification. Chlorophyll–Cu(II) (*M.W.* 724.15) and α-chymotrypsin (*EC 3.421.1*, *M.W.* 25 kDa, USP grade, 1200 U/mg) from bovine pancreas were purchased from Solarbio Inc. (Beijing, China). Cyclen (1,4,7,10-tetraazacyclododecane) was obtained from Kai Sai Inc. (Shanghai, China). Myoglobin (Mb, *M.W.* 16.7 kDa) from equine heart was purchased from Sigma-Aldrich Co., Ltd (St. Louis, MO, US). All chemical reagents were of analytical grade and purchased from local commercial sources. Double-distilled water was used throughout the entire experiment to avoid background interference.

2.2. Preparation of the insoluble catalyst

Chitosan-based Ce(IV) complexes (CC[Ce(IV)]) were synthesized *via* reverse phase suspension crosslinking method and metal ion coordination method (Wang et al., 2013; Kristyna & Ivo, 2013). First, a 5% (w/v) chitosan gel was prepared by introducing acid-dissolved powdery chitosan and 0.5% (w/v) chlorophyll–Cu(II) into 100 mL of 2% (v/v) acetic acid aqueous solution. This mixture was added to 200 mL of liquid paraffin containing 3 mL of Span-80 and stirred at 200 rpm for 30 min. A formaldehyde solution (20 mL, 40% (v/v)) was continuously introduced and reacted for 30 min at 50 °C. After this process, the pH was adjusted to 7.0 using a 0.1 M NaOH solution, followed by the addition of 20 mL glutaraldehyde for 3 h at 60 °C to form chitosan-based matrix particles. The particles were filtered and washed by petroleum ether, ethyl alcohol, and double-distilled water. Subsequently, to activate their functions for sequential modification, the particles were added to 50 mL of *N,N*-dimethylformamide (DMF) while maintaining the pH at 10.0 using a 0.1 M NaOH solution. Then 20 mL of epichlorohydrin was introduced into the above mixture at 60 °C for 3 h. The activated chitosan-based matrix was washed with ethyl alcohol and suspended in 200 mL of DMF containing 5.0×10^{-2} M cyclen, and the modification reaction was carried out at 70 °C for 4 h. Finally, the chitosan–cyclen maxtrix products, termed CC, were filtered from the above mixture and introduced to ethyl alcohol containing 2.0×10^{-2} M Ce(IV). The resulting mixture was incubated at 50 °C for 4 h. When the cyclen–Ce(IV)-modified chitosan-based matrix (CC[Ce(IV)]) was formed, it was collected by filtration and then flushed repeatedly until no metal ions were detected. CC[Ce(IV)] was stored *via* drying in a vacuum afterwards. Further isolation processes were carried out using 20 and 100 mesh sieves to remove large and small irregular fractions, respectively.

2.3. Morphology

According to Wang et al. (2015), microscopic observation of CC[Ce(IV)] microspheres was performed by scanning electron microscope (SEM, JSM-6390LV, JEOL, Japan) and optical microscope (OM, SMZ745T, Nikon, Japan). The dried microspheres were mounted on brass stubs and sputter-coated with gold prior to SEM examination; however, they were directly observed for OM examination. The diameters of the microspheres were measured by a laser particle size analyzer (LPSA, Mastersizer 2000, Malvern Inc., UK). CC[Ce(IV)] microspheres were well diffused in double-distilled water before being loaded in the LPSA.