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Selective cleavage of pepsin by molybdenum metallopeptidase

Sudarat Yenjai, Pinpinat Malaikaew, Teerayuth Liwporncharoenvong, Apinya Buranaprapuk *

Department of Chemistry, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand

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ABSTRACT

In this study, the cleavage of protein by molybdenum cluster is reported for the first time. The protein target used is porcine pepsin. The data presented in this study show that pepsin is cleaved to at least three fragments with molecular weights of ∼23, ∼19 and ∼16 kDa when the mixture of the protein and ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) was incubated at 37 °C for 24 h. No self cleavage of pepsin occurs at 37 °C, 24 h indicating that the reaction is mediated by the metal ions. N-terminal sequencing of the peptide fragments indicated three cleavage sites of pepsin between Leu 112-Tyr 113, Leu 166-Leu 167 and Leu 178-Asn 179. The cleavage reaction occurs after incubation of the mixture of pepsin and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) only for 2 h. However, the specificity of the cleavage decreases when incubation time is longer than 48 h. The mechanism for cleavage of pepsin is expected to be hydrolytic chemistry of the amide bonds in the protein backbone.

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1. Introduction

Selective cleavage of peptides and proteins is an important procedure in biochemistry and molecular biology since the peptide bond is extremely unreactive towards hydrolysis (the half-life for the hydrolysis of the peptide bonds is 7–600 years at room temperature and pH 4–8) [1]. Because uncatalyzed hydrolysis of peptides is extremely slow, artificial cleavage methods are needed. Metal complexes have been attached to specific sites on proteins and subsequent activation resulted in protein fragmentation *via* oxidative or hydrolytic chemistry [2–6]. In oxidative pathway, oxygen-derived radicals have been shown to react with a wide variety of small biological molecules, including amino acids and proteins. The generation of hydroxyl radical has been documented as path of this mechanism. Damage of biological macromolecules by hydroxyl radicals is also a subject of ongoing interest. However, hydrolytic chemistry is preferred over oxidative chemistry as the latter can potentially damage the protein side chains and the resulting fragments may not be amenable to sequencing.

Metal-promoted hydrolytic cleavage of the proteins with a high specificity under mild conditions will be useful for structure–activity studies of proteins, investigation of protein structural domains, and in converting large proteins into smaller fragments that are more amenable for sequencing [7–15]. Many researchers have demonstrated metal-promoted hydrolysis of peptides with metal ions such as palladium(II), platinum(II), copper(II) and zinc(II) [16–21]. Hydrolytic cleavage of lysozyme by Co(III) pentamminea quocobalt(III) (CoPA) and tetramminediaquocobalt(III) (CoTA) ions has been reported previously [22]. Synthetic peptidases could be helpful in elucidating the role of metal ions in natural hydrolases. Although many hydrolases utilize metal ions, the precise role of the metal ion in the hydrolysis reactions is not known. However, specific cleavage of proteins by molybdenum has not yet been reported. Artificial metallopeptidase activity of ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), under mild conditions (37 °C, pH 7), is demonstrated here for the first time. Ammonium heptamolybdate tetrahydrate is a metal cluster with six molybdenums linked together with oxygen. The structure of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ is shown in Scheme 1.

Molybdenum is one of the important metals found in metalloenzymes, such as nitrate reductase [23]. Molybdenum is the only 4d element with a biological function. The physiologically active oxidation states of molybdenum are from +IV to +VI. The compounds found under these conditions involve O ligand, and an important biological function is to catalyze the transfer of oxygen to a two electron substrate. This study will provide a better understanding of the role of metal ions in natural peptidases and in addition aid in the development of more efficient synthetic hydrolases. The gastric aspartic proteinase pepsin (porcine pepsin) was chosen for the current study because the complete amino-acid sequence of porcine pepsin and its three-dimensional structure are known [24–27]. Porcine pepsin is a zymogen-derived protein that has been the subject of extensive studies [28,29]. The enzyme molecule contains 327 residues in a single polypeptide chain. For the current study, the cleavage of pepsin by ammonium heptamolybdate tetrahydrate without adding reducing agents has been investigated.

* Corresponding author. Fax: +66 2 259 2097.
E-mail address: apinyac@swu.ac.th (A. Buranaprapuk).

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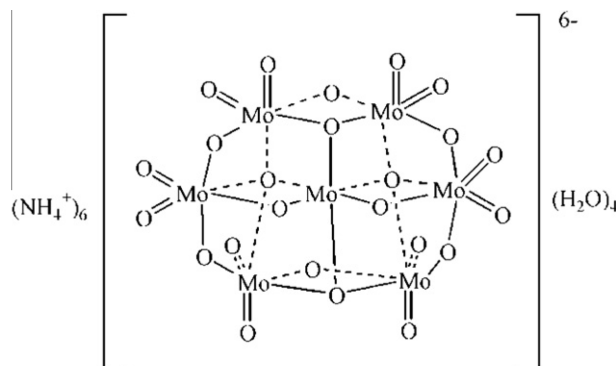


Scheme 1. The structure of ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$).

2. Materials and methods

All materials were reagent grade and were used without further purification unless otherwise noted. Porcine pepsin (Mol. Wt. = 34,623) was purchased from Sigma Chemical Co. Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) was purchased from Sigma Chemical Co. All solutions were freshly prepared in 50 mM Tris–HCl buffer, pH 7.0.

2.1. Protein cleavage conditions

The protein cleavage was carried out at 37 °C in the water bath. The protein solution (15 μM) was treated with $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.125 mM) in 50 mM Tris–HCl buffer, pH 7.0 (total volume 200 μL), and the reaction mixtures were incubated at 37 °C in the water bath for 24 h, unless stated otherwise. The incubated sample was kept frozen afterward. Heat control sample was prepared under the same conditions, as described above, except that the solution was kept frozen after mixing. All reaction mixtures were lyophilized (freeze-drying) until dryness. Incubation time was varied from 2 to 72 h and the incubation temperature was varied to 45 and 55 °C.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were electrophoresed following the literature procedure with minor modifications [30]. Loading buffer (24 μL) containing SDS (7% w/v), glycerol (13% v/v), Tris–HCl (50 mM, pH 6.8), mercaptoethanol (2% v/v) and bromophenol blue (0.01% w/v), was added to protein samples that have been previously dried. Protein solutions (8 μL) were heated for 3 min before loading on the gel. The gels (10%) were run by applying 60 V until the dye passed through the stacking gel. The voltage was then increased to 110 V, as described in the previous report [31]. The gels were run for 1.5 h, stained with Coomassie brilliant blue, and destained in acetic acid solution (10%).

2.3. Peptide transfer and amino acid sequencing

The separated peptide fragments on SDS–polyacrylamide gel were transferred to PVDF membrane with a current of 60 mA for 1.5 h using the semi-dry system (BIORAD) with CAPS buffer, pH 10.5. The transferred protein fragments on PVDF membrane were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% acetic acid). The desired bands were cut and sent for N-terminal amino acid composition analysis (Midwest Analytical, Inc., MO, USA). Chemical sequencing was performed on an automated protein sequencer. Five cycles were performed to identify the N-terminus of the cleaved fragments.

3. Results and discussion

3.1. Cleavage of protein backbone

Pepsin was successfully cleaved by molybdenum cluster at mild conditions and low metal ion concentration (0.125 mM). The protein cleavage was monitored in gel electrophoresis experiments under denaturing conditions. Incubation of pepsin (15 μM) with $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.125 mM) at 37 °C for 24 h resulted in facile cleavage of the protein as demonstrated in SDS–PAGE experiments (Fig. 1). The protein was cleaved resulting in at least three fragments (I, II and III) of molecular weights ~23, ~19 and ~16 kDa, respectively (lane 3). No cleavage was observed in the absence of heat (heat control, lane 2). The reducing agents were not required for this reaction. Self cleavage of pepsin at 37 °C was examined in the absence of the metal complex (data not shown). No reaction occurs at 37 °C, 24 h indicating that the reaction is mediated by the metal cluster.

3.2. Influence of temperature and time of incubation to the cleavage of protein

The reaction rate was not accelerated with temperature when the incubation temperature was varied to 45 and 55 °C (data not shown). The cleavage of pepsin occurred after incubation of the protein and the metal cluster for 2 h (Fig. 2). Increasing time of incubation (2–24 h) increased the product yields and the reaction seemed to become saturated after incubation for 18 h. However, the cleavage became much less specific after incubation longer than 48 h, as seen by smeared bands on the gel (lanes 9 and10).

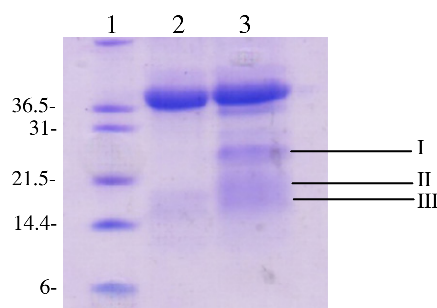


Fig. 1. SDS–PAGE of the thermal reaction products of pepsin by $(NH_4)_6Mo_7O_{24}.4H_2O$. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2–3 contained pepsin (15 μM) and $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.125 mM). (Lane 2 was the heat control while sample in lane 3 was incubated at 37 °C for 24 h.).

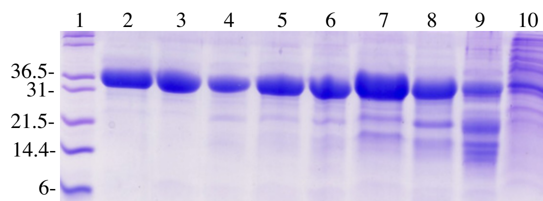
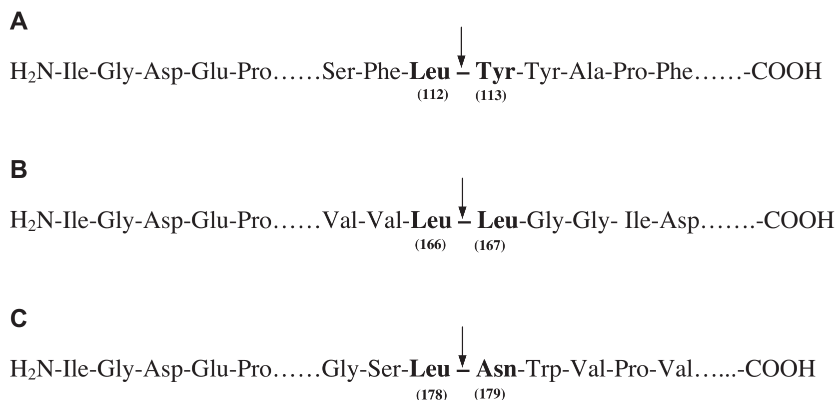
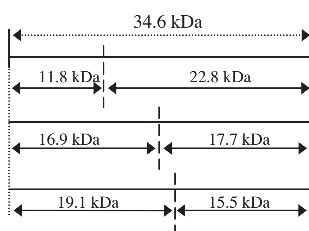


Fig. 2. SDS–PAGE of the thermal reaction products of pepsin by $(NH_4)_6Mo_7O_{24}.4H_2O$. Lane 1 contained molecular weight markers as indicated (kDa). Lane 2 contained pepsin (15 μM). Lanes 3–10 contained pepsin (15 μM) and $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.125 mM). (Lane 3 was the heat control while samples in lanes 4–10 were incubated at 37 °C for 2, 4, 6, 18, 24, 48 and 72 h, respectively.).



Scheme 2. The cleavage sites of pepsin.



Scheme 3. Six cleaved fragments of pepsin with molecular weight of each fragment.

3.3. Amino acid sequencing and cleavage sites determination

To locate the cleavage site on pepsin, the peptide fragments from the gels were isolated and subjected to amino acid sequencing. The newly generated N-terminal sequences of the cleaved fragments were successfully sequenced. N-terminal sequencing of fragment I indicated the residues YYAPF, a sequence internal to pepsin. N-terminal sequencing of fragment II showed two amino acid sequences of the residues LGGID and IGDEP, which the latter is the known N-terminal sequence of pepsin. N-terminal sequencing of fragment III indicated the residues NWVPV, another sequence internal of pepsin. Each newly formed N terminus is sequencible, and the amino acids at the cleavage sites are not modified. The results give clear information in the identification of the cleavage sites. From the known sequence of pepsin, we deduce that the cleavage occurs at Leu 112-Tyr 113, Leu 166-Leu 167 and Leu 178-Asn 179 (Scheme 2).

Reaction of pepsin with $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ appears to produce six fragments which one of them is undetectable on the gel (~12 kDa). The mass of each fragment, calculated from the masses of amino acids in each fragment on the known sequence of pepsin, are close in sum to ~34.6 kDa, the mass of intact pepsin. The data suggest that each pair of fragments is likely to have been formed by a single backbone cut, and there are three cuts occurring in this reaction (Scheme 3).

Selective cleavage of peptide bond requires the formation of the complex in which the metal ion can approach the scissile amide bond. The mechanism for cleavage of pepsin is expected to be hydrolytic chemistry of the amide bonds in the protein, although the mechanism is still not fully elucidated. The mechanism for protein cleavage by Mo(VI) is expected to be similar to that reported for amide hydrolysis by Co(III) [22]. Water molecules bound to the metal ion may participate in acid–base catalysis at the metal binding site, facilitating peptide bond cleavage. Surprisingly, the cleavage occurs at the carboxyl terminal of leucine residues in the cleavage sites. Further studies are needed to investigate the mechanism of this reaction.

From the previous study of amide hydrolysis of Co(III), we have developed our work using a metal cluster instead of a single active metal ion. For our knowledge, this is the first example to show cleavage of protein by a metal cluster. The advantage of using a metal cluster is that a lower concentration of active chemical and a shorter time are required for the protein cleavage reaction. In this study, only 0.125 mM of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ are used, and time of incubation for 2 h is sufficient for the cleavage reaction to occur. This is due to the more active metal ions in the cluster that could speed up the cleavage reaction. Moreover, lower concentration of the metal compound could be a benefit for medicinal process since it causes less harmful to cells.

In conclusion, we have demonstrated that Mo(VI) cluster with six molybdenum cations has the ability to cleave protein under mild conditions without reducing agents. The reaction undergoes possibly *via* a hydrolytic mechanism. The cleavage efficiency reduces when time of incubation was longer than 48 h. This is the first demonstration of protein cleavage by a molybdenum cluster. Chemical proteases derived from inorganic materials may prove to be useful as tools in molecular biology.

Acknowledgment

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