

See discussions, stats, and author profiles for this publication at:
<https://www.researchgate.net/publication/22898108>

Studies on the interaction between Streptomyces pepsin inhibitor and several acid proteinases by means of a zinc(II)-dye complex as a probe

ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · JULY 1975

Impact Factor: 4.66 · DOI: 10.1016/0005-2744(75)90266-1 · Source: PubMed

CITATIONS

19

READS

8

5 AUTHORS, INCLUDING:



Kohei Oda

Kyoto Institute of Technology

166 PUBLICATIONS 2,457

CITATIONS

SEE PROFILE

BBA 67508

STUDIES ON THE INTERACTION BETWEEN *STREPTOMYCES* PEPSIN INHIBITOR AND SEVERAL ACID PROTEINASES BY MEANS OF A ZINC(II)-DYE COMPLEX AS A PROBE

HIROSHI NAKATANI^a, KEITARO HIROMI^a, SHUZO SATOI^b, KOHEI ODA^b,
SAWAO MURAO^b and EIJI ICHISHIMA^c

^aDepartment of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, ^bDepartment of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka and ^cLaboratory of Microbiology and Enzymology, Faculty of Agricultural Science, Tokyo Noko University, Fuchu, Tokyo (Japan)

(Received December 30th, 1974)

Summary

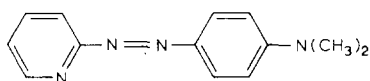
The zinc(II) complex of pyridine-2-azo-*p*-dimethylaniline is bound to several acid proteinases, at pH 5.0, accompanied by a change in the visible absorption spectrum. *Streptomyces* pepsin inhibitor, which was discovered by Satoi and Murao (Satoi, S. and Murao, S. (1970) *Agric. Biol. Chem.* 34, 1265–1267 and Satoi, S. and Murao, S. (1971) *Agric. Biol. Chem.* 35, 1482–1487), is also bound to acid proteinases. Spectrophotometric studies with ten acid proteinases from different sources have revealed that in several acid proteinases, zinc(II)-pyridine-2-azo-*p*-dimethylaniline is released from the enzyme by the inhibitor, while some acid proteinase forms a quaternary complex, zinc(II)-pyridine-2-azo-*p*-dimethylaniline-inhibitor-enzyme. It is speculated that zinc(II)-pyridine-2-azo-*p*-dimethylaniline is bound to two catalytic carboxylate groups in the active site of the acid proteinases and the inhibitor is bound mainly to the substrate-binding site of the enzymes. The binding of the inhibitor may overlap the catalytic site completely or partially. The degree of overlapping is characteristic of the kind of acid proteinases.

Introduction

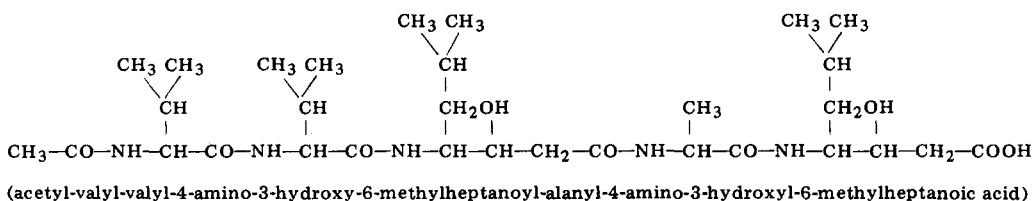
In 1954, Klotz and Ming [1] pointed out from the change in the visible absorption spectrum that the azo dye, pyridine-2-azo-*p*-dimethylaniline(PAD), which cannot be bound to pepsin by itself, can be bound in the presence of

certain transition metal ions such as zinc(II). The proposed model was the metal-mediated mixed complex, (dye)-(metal ion)-(pepsin) [1], but neither the detailed structure of the mixed complex nor the binding site of the metal ion in the pepsin molecule have been elucidated. Our preliminary experiments showed that some other acid proteinases also exhibit the same spectral behaviour as pepsin at pH 5. However, lysozyme (hen egg white), amylases (liquefying α -amylase, and glucoamylase), and neutral or alkali proteinases such as α -chymotrypsin or subtilisin did not show any such spectral change at pH 5. Thus, the formation of the mixed zinc(II)-PAD-enzyme complexes seems to be specific to certain acid proteinases.

On the other hand, there have been found two kinds of pepsin inhibitors which are very similar in structure, *Streptomyces* pepsin inhibitor by Sato and Murao [2,3] and pepstatin by Umezawa et al. [4]. The inhibiting specificity of these inhibitors towards various acid proteinases has been studied [3,4]. In this paper, we aimed to obtain information about the structure of the active site of several acid proteinases through the interaction of *Streptomyces* pepsin inhibitor with the enzymes using the zinc(II)-PAD complex. The interaction between the inhibitor and acid proteinases was also studied directly by ultraviolet difference spectrophotometry. The results showed that the ten acid proteinases studied can be classified into three groups according to their behaviour toward the inhibitor and the zinc(II)-PAD complex.



Pyridine - 2 - azo - *p* - dimethylalanine



Streptomyces pepsin inhibitor

Materials and Methods

Three-times recrystallized pepsin (EC 3.4.23.1) was purchased from Miles. Acid proteinase from *Rhizopus chinensis* (EC 3.4.23.9), acid proteinase A and B from *Aspergillus niger* (EC 3.4.23.6) were purchased from Seikagaku Kogyo Co. Ltd. Acid proteinases from *Rhodotorula* [5,6], *Cladosporium* [7], *Aspergillus saitoi* (EC 3.4.23.6) [8], *Scytalidium lignicolum* (type AI, AII, and B) [9,10] and *Streptomyces* pepsin inhibitor [2,3] were prepared as described elsewhere. Zinc sulfate and PAD were obtained commercially from Nakarai Co. Ltd. All sample solutions were prepared with 0.05 M acetate buffer (pH 5.0). Visible spectra were measured with a Shimadzu UV 200 spectrophotometer at 25°C. The proteinase activity was measured by the modified Anson's method [11].

Results

Zinc(II)-PAD complex has an absorption maximum at about 530 nm at pH 5.0, and increases its absorbance in the presence of pepsin or other acid proteinases. Fig. 1 shows the spectral change in the case of pepsin and acid proteinase A from *A. niger*. In the absence of zinc(II), however, spectral change caused by the addition of enzymes was very small or negligible except for type B of *Scytalidium* which showed some change in the dye's absorption spectrum. *Streptomyces* pepsin inhibitor has been known as a competitive inhibitor for several kinds of acid proteinases [14]. The spectral behavior caused by the addition of the inhibitor to zinc(II)-PAD-enzyme solutions are classified into three groups depending on the kind of acid proteinases.

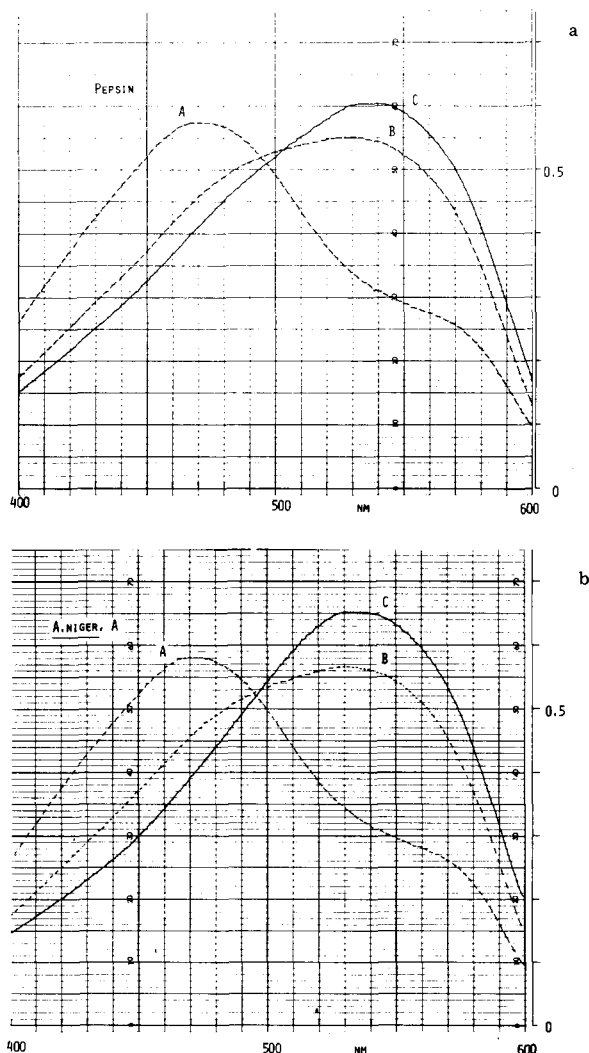


Fig. 1. Absorption spectra of PAD, zinc(II)-PAD, and zinc(II)-PAD-enzyme at pH 5.0 (0.05 M acetate buffer). (a) pepsin, (b) *A. niger* (type A) acid protease. A: PAD alone ($2.89 \cdot 10^{-5}$ M), B: PAD plus ZnSO_4 ($2 \cdot 10^{-3}$ M) C: PAD plus ZnSO_4 and the enzyme (1 mg/ml).

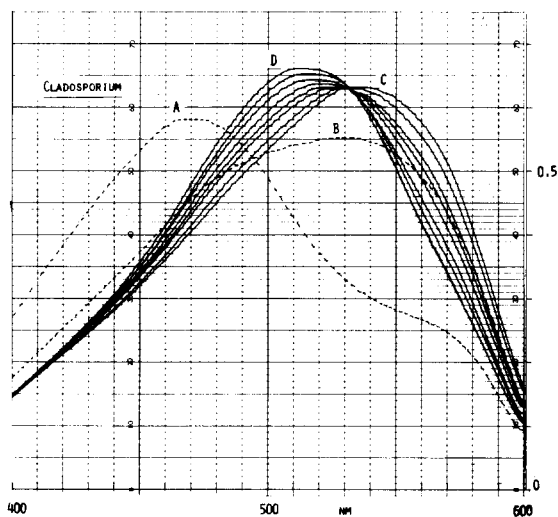


Fig. 2. Absorption spectra of zinc(II)-PAD-*Cladosporium* acid proteinase-inhibitor system. The meaning of A, B, and C are the same as those in Fig. 1. D is the absorption spectrum of zinc(II)-PAD-enzyme-inhibitor at the large excess of the inhibitor. The spectrum C shifts to the spectrum D upon addition of the inhibitor.

- (1) The absorption spectrum characterized by zinc(II)-PAD-enzyme mixed complex decreases and approaches the initial absorption spectrum of zinc(II)-PAD.
- (2) The absorption spectrum shifts to the shorter wavelength, which indicates the existence of quaternary zinc(II)-PAD-inhibitor-enzyme complex.
- (3) No spectral change is brought about by the inhibitor.

The enzymes belonging to Class 1 are pepsin, acid proteinases from *R. chinensis*, from *Rhodotorula*, and from *A. saitoi*, and acid proteinase B from *A. niger*. To Class 2 belongs acid proteinase from *Cladosporium*; the spectral changes are shown in Fig. 2. Class 3 involves acid proteinase A from *A. niger*, and acid proteinase (type AI, AII, and B) from *Scytalidium*. The titration of

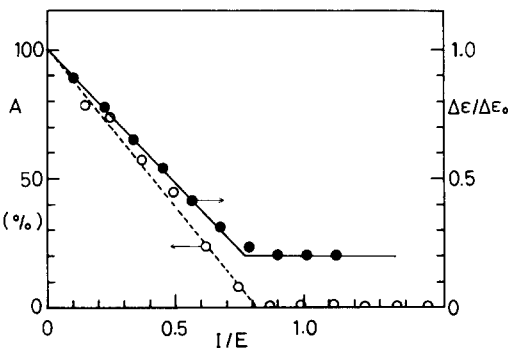


Fig. 3. Titration of pepsin with *Streptomyces* pepsin inhibitor by the inhibition of enzyme activity and visible absorbance change at 570 nm. A: Enzyme activity, measured at pH 2.0 (enzyme concentration, $6.35 \cdot 10^{-6}$ M). $\Delta\epsilon/\Delta\epsilon_0$: Relative absorbance change at 570 nm and pH 5.0 (enzyme concentration $2.25 \cdot 10^{-5}$ M). The solution contains PAD ($2.89 \cdot 10^{-5}$ M) and ZnSO_4 ($2 \cdot 10^{-3}$). I/E: Inhibitor-enzyme ratio.

TABLE I

SUMMARY OF THE PROPERTIES OF ACID PROTEINASES FROM VARIOUS SOURCES TOWARD SEVERAL REAGENTS

Positive and negative effects are shown by the symbols + and - respectively. Evidence for *Streptomyces* pepsin inhibitor (SPI) binding is obtained from the appearance of an ultraviolet difference spectrum.

Enzyme	Mol. wt	Zn-(PAD) ²⁺ binding	SPI binding	Release of Zn-(PAD) ²⁺ by SPI	Inhibition by SPI	Inactivation by diazoacetyl- norleucine methylester
Pepsin	35000	+	+ [12]	+	+ [3]	+ [16]
<i>Rhodotorula</i>	32000	+	+ [14]	+	+ [3]	+ [14]
<i>R. chinensis</i>	34000	+	+*	+	+ [15]	+ [15]
<i>A. saitoi</i>	35000	+	+*	+	+	+ [17]
<i>A. niger</i> (type B)	34000	+	+*	+	+	+ [17]
<i>Cladosporium</i>	30000	+	+ [14]	-	+ [3]	+ [14]
<i>A. niger</i> (type A)	19000	+	-*	-	-	- [18]
<i>Scytalidium</i> (type AI)	40000	+	- [9]	-	- [9,10]	- [18]
<i>Scytalidium</i> (type AII)	40000	+	- [9]	-	- [9,10]	- [18]
<i>Scytalidium</i> (type B)	21000	+	+ [9]	-	- [9,10]	+ [18]

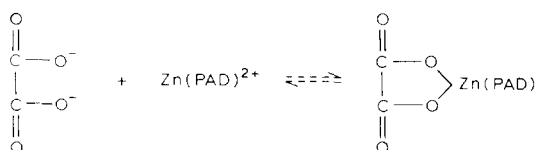
* Nakatani, H., unpublished data.

pepsin with the inhibitor using the spectral change at 570 nm is shown in Fig. 3. The residual absorbance was always detected even in the large excess of the inhibitor. This is probably due to the inactive enzyme and/or residual minor binding sites for zinc(II)-PAD which cannot interact with the inhibitor. The titration by proteinase activity shows the same behaviour as the spectroscopic titration (Fig. 3). Binding of *Streptomyces* pepsin inhibitor to acid proteinases could be detected directly by ultraviolet difference spectrophotometry. Difference spectra caused by the inhibitor always appeared for the enzymes of Classes 1 and 2 (refs 12 and 14 and Nakatani, H., unpublished data). No difference spectrum was observed for the enzymes of Class 3, except type B from *Scytalidium* which shows a difference spectrum with a large excess of the inhibitor [9]. The results together with some other characteristics of the enzymes are summarized in Table I.

Discussion

It has been established that the catalytic function of pepsin is performed by two carboxyl groups interacting with each other [13]. It would be a reasonable statement that the catalytic site of acid proteinases of microbial source is also composed of at least two carboxyl groups, judging from the similarity in optimum pH and reactivity towards chemical modifiers between pepsin and acid proteinases of microbial source. Most acid proteinases are inactivated by esterification of the carboxyl groups by diazoacetylnorleucine methylester, but the acid proteinases of types AI, AII, and B from *Scytalidium* are not inactivated by this chemical; these enzymes are considered to be acid proteinases of a new type [11] (Table I). The spectral change of zinc(II)-PAD caused by acid proteinase (Fig. 1) is very similar to that by oxalic acid. It is quite reasonable to consider that the spectral change of zinc(II)-PAD by the

addition of oxalic acid is due to the formation of the mixed complex of the type



Since the spectral change of zinc(II)-PAD, caused by the addition of the acid proteinases, is the same as that observed with oxalic acid and since two carboxyl groups as catalytic residues may be common for most acid proteinases, it is reasonable to consider that zinc(II)-PAD binds to the catalytic residues in acid proteinases, forming a mixed chelate complex. However, the concentration of oxalic acid necessary to cause the same spectral change is about 100 times larger than that of acid proteinases (Nakatani, H., unpublished).

As *Streptomyces* pepsin inhibitor inhibits the activity of many acid proteinases and protects them from esterification of the carboxyl group in the catalytic site, it is clear that the inhibitor binds at the active site, probably the substrate-binding site, of acid proteinases. If the inhibitor completely covers the catalytic site, zinc(II)-PAD coordinating to the catalytic carboxyl groups may be replaced by the inhibitor. This is just what has been seen in the case of Class 1 enzymes. If the inhibitor covers the catalytic site partially, zinc(II)-PAD may still be bound simultaneously on the active site of acid proteinases, then Class 2 enzyme, *Cladosporium* acid proteinase, would be the case. The Class 3 enzyme, which cannot bind the inhibitor on the active site, is only able to bind zinc(II)-PAD on the catalytic site. Although type B from *Scytalidium* binds the inhibitor, causing ultraviolet difference spectra [9], zinc(II)-PAD is not released from the enzyme and its proteolytic action is not inhibited by the inhibitor [9]. Therefore the binding site for the inhibitor in this enzyme must be far from the catalytic site.

Finally, the following consideration may be possible; as the zinc(II) ion tends to form tetrahedral or octahedral complexes, the distance between two oxygen atoms of the catalytic carboxylates, to which the zinc(II)-PAD coordinates, is about 3 Å, using the data from the geometrical coordination structure of zinc(II) complexes [19].

References

- 1 Klotz, I.M. and Ming, W.-C.L. (1954) *J. Am. Chem. Soc.* **76**, 805-814
- 2 Sato, S. and Murao, S. (1970) *Agric. Biol. Chem.* **34**, 1265-1267
- 3 Sato, S. and Murao, S. (1971) *Agric. Biol. Chem.* **35**, 1482-1487
- 4 Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, H., Hamada, M. and Takeuchi, T. (1970) *J. Antibiot.* **23**, 259-262
- 5 Kamata, K., Oda, K. and Murao, S. (1972) *Agric. Biol. Chem.* **36**, 1103-1108
- 6 Kamata, K., Oda, K. and Murao, S. (1972) *Agric. Biol. Chem.* **36**, 1095-1101
- 7 Murao, S., Funakoshi, S. and Oda, K. (1972) *Agric. Biol. Chem.* **36**, 1327-1333
- 8 Ichishima, E. (1970) in *Methods in Enzymology* (Perlmann, G.E. and Lorand, L., eds), Vol. XIX, pp. 397-406, Academic Press, New York
- 9 Murao, S., Oda, K. and Matsushita, Y. (1972) *Agric. Biol. Chem.* **36**, 1647-1650

- 10 Murao, S., Oda, K. and Matsushita, Y. (1972) *Agric. Biol. Chem.* 37, 1417-1421
- 11 Akabori, S. (1956) in *Methods of Enzyme Research*, Vol. 2, pp 237-293, Asakura Book Co. Ltd, Tokyo (in Japanese)
- 12 Sato, S. and Murao, S. (1972) *Agric. Biol. Chem.* 36, 515-518
- 13 Fruton, J.S. (1971) in *The Enzymes* (Boyer, P.D., ed.), Vol. 3, pp. 120-164, Academic Press, New York
- 14 Sato, S. (1974) Ph. D. Thesis, University of Osaka Prefecture
- 15 Tsuru, D., Fujimura, K., Yoshimoto, T., Watanabe, R., Tomomatsu, M. and Hayashida, S. (1973) *Int. J. Protein Res.* 5, 293-295
- 16 Rajagopalan, T.G., Stein, W.H. and Moore, S. (1966) *J. Biol. Chem.* 341, 4295-4297
- 17 Takahashi, K. and Chang, W.-J. (1973) *J. Biochem.* 73, 675-677
- 18 Oda, K. and Murao, S. (1974) *Agric. Biol. Chem.* 38, 2435-2444
- 19 Freeman, H.C. (1973) in *Inorganic Biochemistry* (Eichorn, G.L., ed.), Vol. 1, pp 121-166, Elsevier, Amsterdam