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Enzymatic oxidation of the bifunctional wheat inhibitor of subtilisin and endogenous α-amylase

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Oxidation of the bifunctional wheat inhibitor of subtilisin and endogenous α-amylase catalyzed by horseradish peroxidase results in the loss of the inhibitory activity against both enzymes. The enzymatic oxidation is accompanied by modification of one methionine and two tryptophan residues in the protein. The results obtained, together with data on chemical modification and limited proteolysis, allow us to conclude that Met^{34} Ala^{35} is the reactive site of the inhibitor responsible for the interaction with subtilisin. It is supposed that the reactive site of the inhibitor responsible for the interaction with α-amylase contains one or two tryptophan residues.

Enzymatic oxidation; Bifunctional inhibitor; Subtilisin; α-Amylase

1. INTRODUCTION

Bifunctional proteins able to inhibit the activity of both endogenous germination-specific α-amylase of seeds and microbial serine proteinases were isolated from seeds of some cereals [1–6]. All these proteins belong to the soybean trypsin inhibitor (Kunitz) family. The inhibitors contain two separate sites for binding of proteinase and α-amylase [1,7]. The wheat inhibitor contains the critical methionine residue, modification of which results in the loss of the inhibitory activity against subtilisin [7,8]. At the same time, X-ray structure analysis showed that the molecule of the bifunctional inhibitor contained Gly^{66}–Ala^{67} residues at the position corresponding to Arg^{64}–Ile^{65} of the reactive site of the soybean trypsin inhibitor. Nevertheless, on incubation of the inhibitor with proteinase K, the peptide bond between Ile^{75} and Ser^{76} is hydrolyzed [9].

In order to obtain additional information on the structure of the reactive sites, the bifunctional wheat inhibitor has been subjected to oxidation catalyzed by horseradish peroxidase. The results obtained, together with our earlier data on chemical modification and limited proteolysis [7,10], allow us to conclude that one methionine residue occupies the P1 position of the reactive site responsible for the interaction with subtilisin. It is suggested that the reactive site responsible for the interaction with α-amylase contains one or two tryptophan residues.

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2. MATERIALS AND METHODS

The inhibitor was isolated from wheat seeds using affinity chromatography on immobilized subtilisin [5]. Subtilisin Carlsberg and barley malt α-amylase II were purchased from Sigma Chemicals Co., and horseradish peroxidase was a product of Reanal Co.

To assay inhibitor activity, increasing amounts of the inhibitor were added to medium containing a constant amount of subtilisin or α-amylase. The mixture was incubated for 5 min at room temperature, and then the residual enzyme activity was determined. The subtilisin activity was measured using N-CBZ-L-Ala-L-Ala-L-Leu *p*-nitroanilide [11], and the α-amylase activity was evaluated using amylopectin azure as substrate [12].

The enzymatic oxidation of the inhibitor was performed as follows. The protein was incubated with optimal concentrations of horseradish peroxidase in the presence of H_2O_2 and I^- for 10 min at 37°C and pH 6.6, and then NaN_3 was added to stop the reaction. The oxidized inhibitor was separated by chromatography on a Sephadex G-75 column.

The inhibitor–subtilisin complex was prepared by mixing the inhibitor with equimolar amounts of subtilisin in Tris-HCl buffer, pH 8.0. After oxidation with horseradish peroxidase the inhibitor–subtilisin complex was dissociated by adjusting the pH to 4.0. Under these conditions, complete inactivation of the released subtilisin occurs.

The amino acid composition of the inhibitor was determined after hydrolysis with 5.7 N HCl for 24 h at 110°C. Cysteine and methionine were determined after oxidation with performic acid. To determine tryptophan, the inhibitor was hydrolyzed with methane-sulfonic acid.

3. RESULTS AND DISCUSSION

The oxidation of the bifunctional wheat inhibitor catalyzed by horseradish peroxidase in the presence of iodide ions results in a loss of inhibitory activity against both subtilisin and barley α-amylase II (Fig. 1). When I^- was replaced with Cl^-, no inactivation of the inhibitor was observed. The degree of oxidation depended on the concentration of the components of the peroxidase–H_2O_2–I^- system, and, in the absence of any of them, no

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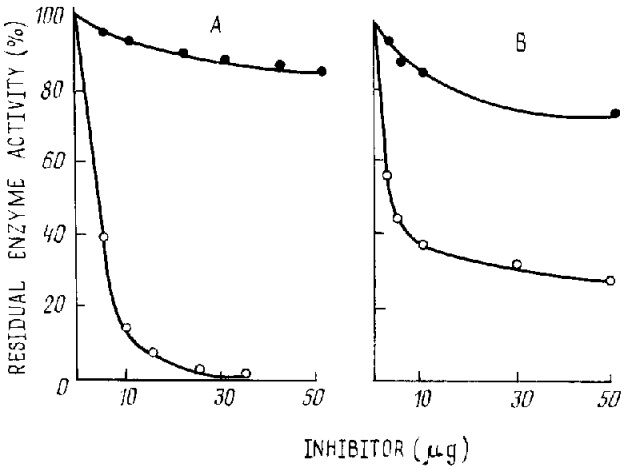

Fig. 1. Inhibition of subtilisin (A) and α-amylase (B) with the bifunctional wheat inhibitor. Native inhibitor (○); inhibitor subjected to enzymatic oxidation (●).

oxidation occurred. The amino acid assay of the oxidized protein (Table I) showed that the enzymatic oxidation is accompanied by modification of one of the two methionine and two tryptophan residues. Thus, horseradish peroxidase oxidizes the same amino acid residues in proteins as does myeloperoxidase [14]. In contrast to horseradish peroxidase, however, myeloperoxidase was active in the presence of both I^- and Cl^- ions.

As was shown earlier, the treatment of the bifunctional wheat inhibitor with monoiodoacetic acid and chloramine T under conditions causing modification of methionine residues in proteins was accompanied by the loss of activity against subtilisin [5,7]. On this basis we may conclude that the loss of the inhibitory activity against subtilisin on oxidation catalyzed by horseradish peroxidase is a result of modification of the methionine residue.

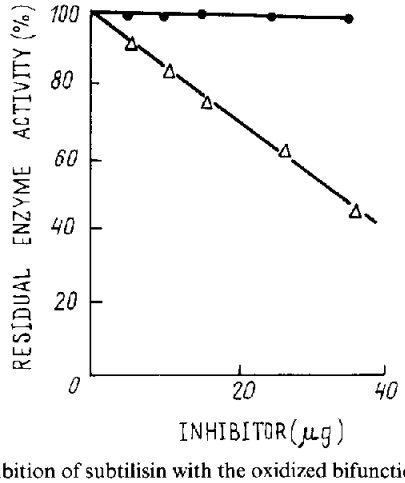

Fig. 2. Inhibition of subtilisin with the oxidized bifunctional inhibitor. Inhibitor subjected to the enzymatic oxidation in the free state (●); inhibitor subjected to the enzymatic oxidation in the complex with subtilisin followed by dissociation in the acidic medium (△).

The activity of the inhibitor subjected to the enzymatic oxidation in the complex with subtilisin remains at a rather high level and is easily detected after dissociation of the inhibitor–subtilisin complex in an acidic medium (Fig. 2). Thus, all the results obtained support the assumption that the reactive site of the inhibitor contains a methionine residue. In previous work, we isolated a fragment with a high inhibitory activity against subtilisin from the inhibitor hydrolysed with staphylococcal protease V8 [10]. The fragment was found to be the N-terminal part of the inhibitor molecule and contained a Met^{34} residue (based on the primary structure determined by Maeda [15]). On the basis of the aforesaid evidence, we may conclude that the reactive site of the bifunctional wheat inhibitor includes Met^{34} Ala^{35}. It is noteworthy that the methionine residue at the P1 position was detected in the reactive sites of a number of protease inhibitors of microbial serine proteinases [16–18]. As follows from the results obtained, the ability of methionine residues to be enzymatically oxidized under mild conditions is a possible way to regulate proteolytic activity.

There is little evidence for the composition and structures of the reactive sites of bifunctional inhibitors responsible for the interaction with α-amylase. In our previous works we have shown that modification of methionine residues in the inhibitor molecule has no effect on its ability to inhibit α-amylase [7,8]. Thus, the loss of activity against α-amylase on oxidation catalyzed by horseradish peroxidase (Fig. 1B) seems to result from modification of one or both tryptophan residues in the protein molecule. It is known that in some microbial inhibitors of α-amylase, there is a critical tryptophan residue in the conserved sequence Trp-Arg-Tyr [19]. In the bifunctional inhibitor of subtilisin and

Table I

Amino acid composition of the bifunctional wheat inhibitor

	Native	Oxidized
Asp	17.2	17.1
Thr	8.4	8.2
Ser	10.7	10.9
Glu	12.3	12.0
Pro	14.3	14.0
Gly	18.8	19.3
Ala	15.5	15.9
1/2 Cys	3.9	4.3
Val	13.3	13.4
Met	1.7	0.9
Ile	6.7	6.6
Leu	11.0	11.0
Tyr	4.7	4.5
Phe	5.6	5.9
His	8.1	7.5
Lys	6.4	6.2
Arg	13.9	13.6
Trp	2.1	0.0

The composition has been adjusted to M_r 20,500 for the protein.

endogenous α-amylase from barley, one of the three tryptophan residues is readily oxidized with *N*-bromosuccinimide without any loss of activity against α-amylase, while modification of the other two residues results in the complete loss of this activity [20]. As has been shown above, in the bifunctional wheat inhibitor, both tryptophan residues are readily oxidized in the presence of horseradisch peroxidase and the enzymatic oxidation results in the loss of the inhibitory activity against α-amylase. Thus, we can assume that the reactive site of the bifunctional wheat inhibitor responsible for the interaction with α-amylase contains one or two tryptophan residues.

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REFERENCES

[1] Mundy, J., Svendsen, I. and Hejgaard, J. (1983) Carlsberg Res. Commun. 48, 81 90.
[2] Weselake, R.J., Mac Gregor, A.W., Hill, R.D. and Duckworth, H.W. (1983) Plant Physiol. 73, 1008–1012.
[3] Mundy, J., Hejgaard, J. and Svendsen, I. (1984) FEBS Lett. 167, 210–214.
[4] Jany, K.-D. and Lederer, G. (1985) Biol. Chem. Hoppe-Seyler 366, 807– 808.
[5] Mosolov, V.V. and Sul'gin, M.N. (1986) Planta 167, 595–600.
[6] Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomes, Z., Royo, J. and Carbonero, P. (1987) Oxford Surveys Plant Mol. Cell Biol. 4, 275–334.
[7] Nesterenko, M.V., Mitskevich, L.G. and Mosolov, V.V. (1987) Biochimiya 52, 1665–1669.
[8] Mosolov, V.V. and Gvozdeva, E.L. (1992) Plant Physiol. Biochem. 30, 181 185.
[9] Zemke, K.J., Müller-Fahrnow, A., Jany, K.-D., Pal, G.P. and Saenger, W. (1991) FEBS Lett. 279, 240–242.
[10] Nesterenko, M.V., Gvozdeva, E.L., Mitskevich, L.G. and Mosolov, V.V. (1989) Biochimiya 54, 838–845.
[11] Ljublinskaya, L.A., Jakusheva, L.D. and Stepanov, V.M. (1977) Bioorg. Chem. (USSR) 3, 273–279.
[12] Hall, F.F., Gulp, T.W., Hayakawa, T., Ratliff, C.R. and Hightower, N.C. (1970) Am. J. Clin. Pathol. 53, 627 634.
[13] Simpson, R.L., Neuberger, M.R. and Lin, T.G. (1976) J. Biol. Chem. 251, 1936–1940.
[14] Matheson, N.R., Wong, P.S. and Travis, J. (1979) Biochem. Biophys. Res. Commun. 88, 402–409.
[15] Meada, K. (1986) Biochim. Biophys. Acta 871, 250–256.
[16] Richardson, M., Barker, R.D., Mc Millan, R.T. and Cossins, L.M. (1977) Phytochemistry 16, 837–839.
[17] Omichi, K., Nagura, N. and Ikenaka, T. (1980) J. Biochem. 87, 483 489.
[18] Jonassen, I. and Svendsen, I. (1982) Carlsberg Res. Commun. 47, 199–203.
[19] Pflugrath, J.W., Wiegand, G. and Huber, R. (1986) J. Mol. Biol. 189, 383–386.
[20] Halayko, A.J., Hill, R.D. and Svensson, B. (1986) Biochim. Biophys. Acta 873, 92–101.