ISOLATION AND STUDY OF THE PROTEASE—INHIBITOR SYSTEM OF WHEAT GRAIN

O. V. Fursov

UDC 581.192:557.156.7

The study of the regulation of enzymatic activity at the level of the functioning of the enzymes is one of the main questions of modern enzymology. There is information in the literature on the presence in the seeds of several plants of natural protein inhibitors suppressing the activity of the corresponding proteases [1-6]. Inhibitors of this type played the role of regulators of protease activity.

Polyanovskii's method A [6], developed for rye seeds has proved unsuitable for studying the protease—inhibitor system in germinating wheat grains, since the isolation of the proteases and protein inhibitors was accompanied by a considerable contamination of them by the accompanying albumins of the endosperm. We have suggested a method of the additional purification of the inhibitor and the proteases which has enabled the specific activity of the enzyme to be increased by a factor of 4-5 and the activity of the inhibitor by a factor of 3-4. The modification of the method makes it possible to determine the activity of the inhibitor in the process of the sprouting of the seed. It has been established that the inhibitor of the intrinsic proteases of wheat grain is localized in the endosperm, and in the process of germination of the grain its activity falls. The bulk (about 70%) of the protease activity is also concentrated in the endosperm of the seed. The protease—inhibitor systems of three varieties of soft wheat (Bezostaya 1, Kazakhstanskaya 126, and Dneprovskaya 56) and one type of hard wheat (Khar'kovskaya 46) of the 1973 harvest were studied. For comparison, the activities of the inhibitors (Table 1) were expressed in papain units. The varieties studied are characterized by the specific type of fall in the activity of the inhibitor in the germination of the seeds. The endosperm of the seed of the hard wheat contained inhibitor only in the state of dormancy. The specific activities of the proteases of the types considered changed only slightly in the germination process. This fact shows the complete dissociation of the inhibitor—enzyme complex under the experimental conditions. Variations in the specific activities are due to the presence of ballast proteins in the enzyme fractions.

Electrophoresis of wheat proteases was performed in the presence of a hemoglobin substrate in a gel. In all the varieties, two active isoenzymes of protease were found.

EXPERIMENTAL

The seeds were germinated at 27°C for 12 and 24 h. The endosperm (5 g) of the dormant and germinating seeds were separated and comminuted in a coffee mill. After milling, the endosperm of the germinating seeds was additionally ground in a mortar in the presence of the extractant. Extraction was performed with distilled water (1:10) with constant stirring for 1 h. After centrifuging (12,000 g, 20 min) the pH of the supernatant was brought to 4.5 with 0.1 N HCl, and it was left in the refrigerator overnight. Under these conditions dissociation of the protease—inhibitor complex took place, and some of the ballast proteins precipitated. These proteins were separated off by centrifuging (10,000 g, 15 min). The protein fraction containing the inhibitors was precipitated by half-saturation with ammonium sulfate. The precipitate formed in 2 hours at room temperature, and then it was collected by centrifuging (12,000 g, 15 min) and dissolved in 0.9% NaCl. To the supernatant containing the protease was added ammonium sulfate to 75% saturation and the mixture was left for

Kazakh Scientific-Research Institute of Agriculture. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 642-645, September-October, 1975. Original article submitted June 25, 1974.

© 1976 Plenum Publishing Corporation, 227 West 17th Street, New York, N.Y. 10011. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission of the publisher. A copy of this article is available from the publisher for $15.00.

TABLE 1. Specific Activities of the Proteases and Activities of the Inhibitors of Germinating and Dormant Seeds of Several Types of Wheat

State of the material investigated	Specific activity of the protease	Activity of the inhibitor with respect to the corresponding protease	Activity of the inhibitor, papain units
Dneprovskaya 56			
Dormant	0,114	7,0	0,183
12-hour germination	0,112	8,0	0,210
24-hour germination	0,110	0	0
Bezostaya 1			
Dormant	0,096	7,6	0,500
12-hour germination	0,100	13,0	0,860
24-hour germination	0,090	18,0	1,180
Kazakhstanskaya 126			
Dormant	0,130	19,8	0,583
12-hour germination	0,127	22,0	0,650
24-hour germination	0,126	25,1	0,740
Khar'kovskaya 46			
Dormant	0,125	23,2	0,500
12-hour germination	0,121	0	0
24-hour germination	0,130	0	0

1 h. To a solution of the protein in 0.9% NaCl was added TCA to a final concentration of 2% and the mixture was left in the cold for 1 h. The precipitates that deposited were collected by centrifuging (10,000 g, 15 min) and discarded. To the supernatant containing the inhibitor was added TCA to a final concentration of 12%, and the protease-containing supernatant was brought to 100% saturation with ammonium sulfate. The precipitate of the enzyme formed in the course of 2 h at room temperature; the inhibitor was precipitated in the cold during the same time. Then the inhibitor was collected by centrifuging at 15,000 g for 30 min, and the proteases were precipitated at 12,000 g for 15 min. The inhibitor was dissolved in 0.9% NaCl and the protease in water. To obtain comparable results, the enzyme and inhibitor must be dissolved in strictly constant volumes of solvents. The solutions obtained were dialyzed first against running mains water for 2 h and then against distilled water for 12 h.

The proteolytic activity was determined by a modified method [7]. Incubation was performed in the presence of 0.2 M β-mercaptoethanol at 30°C for 1 h. As the substrate we used a 2% solution of hemoglobin in 0.2 M acetate buffer, pH 5.4. In the precipitation of the protein, instead of 5% TCA we used 20% TCA. Filtration after incubation was replaced by centrifuging for 25 minutes at 15,000 g.

To determine the inhibition, to one part of a solution of protease (as protein) we added 3-4 parts of inhibitor protein and preincubated the mixture for 10 min. The protein was determined by Lowry's method [8]. The specific activity of the intrinsic protease was expressed in μg of tyrosine/mg of protein·min. The optical densities of the solutions were measured at 280 nm in an SF-4A spectrophotometer. The amount of tyrosine was determined from a calibration curve, and the level of inhibition by the difference in the specific activities of the preparation of protease without the inhibitor and with the inhibitors. The activity of the inhibitor was calculated in milligrams of inhibitor protein causing 100% inhibition of 1 mg of the intrinsic enzyme. For comparing the activities of the inhibitors from the different varieties, they were expressed in papain units. For this purpose we determined the capacity of the inhibitor of the dormant endosperm for suppressing the activity of one unit of a standard papain preparation (Fluka A. G. Chemische Fabrik 60,000 E/T). Then we calculated the ratio of the activity of the inhibitor with respect to the intrinsic enzyme in various stages of germination and the activity of the inhibitor with respect to papain.

With the germination of the seeds the amount of inhibitor protein binding 1 mg of enzyme increased, which shows a fall in the activity of the inhibitor. At the same time, the relative values (papain units) rose.

The disk electrophoresis of the proteases was performed in 7.5% acetic acid polyacryl-

amide gel in the presence of 3 M urea and 0.25% hemoglobin. The reservoir liquid was 0.01 M solution of acetic acid. The time of electrophoresis was 50 min. The gels were incubated and were stained with Amido Black. After washing, the protease isoenzymes appeared in the form of light bands on a dark green background.

SUMMARY

A modified method for obtaining a protease and its natural protein inhibitor from the endosperm of wheat grain is proposed. The enzyme is purified by additional precipitation of part of the ballast proteins with ammonium sulfate, and the inhibitor by two successive precipitations of the bulk of the inactive protein with the aid of TCA of different concentrations. The specific activity of the protease increases by a factor of 5-6, and the activity of the inhibitor by a factor of 3-4. Two active isoenzymes of protease have been found in each of the varieties of wheat investigated by disk eletrophoresis.

LITERATURE CITED

1. Y. Shain and A. M. Mayer, Phys. Plant., 18, 853 (1965).
2. E.-M. Soulinna, J. Mikola, and T.-M. Enari, J. Institute Brew., 71, 519 (1965).
3. J. Mikola and T.-M. Enari, J. Institute Brew., 76, 182 (1970).
4. W. C. Burger and H. W. Siegelman, Phys. Plant., 19, 1089 (1966).
5. J. Mikola and E.-M. Suolinna, Arch. Biochem. Biophys., 144, 566 (1971).
6. A. Polanowski, Acta Biochim. Polonica, 14, 389 (1967).
7. S. I. Petrovaya and M. M. Vintsyunaite, Prikl. Biokhim. i Mikrobiol., 2, 322 (1966).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).