

Determination of proteolytic activity in cereals

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Abstract

Results have been presented indicating that native proteolytic enzymes and bromelain acted variously on wheat proteins differentiated in quality, they also act nonspecifically on haemoglobin. It was thought useful to elaborate a new method of proteolytic activity determination based on the application of a standard natural substrate. The latter consists of gluten extract in acetic acid from a definite flour sample. The method of substrate selection and the determination procedure are described and some examples of wheat proteolytic activity determination are given.

INTRODUCTION

Proteolytic activity determination in cereal grains gives information concerning the baking quality. An excess of those enzymes causes a too advanced degradation of structural protein and a low level gives insufficient loosening of gluten, particularly in flour of higher quality. In such a case addition of proteolytic enzymes is even sometimes recommended. Very high activity of those enzymes develops, when germination process is started. Therefore the proteolytic activity should be considered as one of important characteristics of bread grains.

The methods of determination proteolytic activity in flour are rather unprecise, because of the low level of this activity, the heterogeneous mechanisms of enzyme action and their different substrate specificity. Of significance is also the fact of complexing some proteinases with particular gluten subunits (Kamiński, Bushuk, 1969; Kozmina, 1974) suggesting their specificity for those substrates. It is connected with the differentiated active site structure, as among the cereal peptidases SH enzymes (Mac Donald, Chen, 1964; Skupin, War-

chalewski, 1971), as well as metal enzymes were found to exist (Hwang, Bushuk, 1973). The methods applied up till now are based on the liberation of soluble nitrogen and its determination by colorimetric methods (Anson, 1963; MacDonald, Chen, 1965). Anson's most popular method uses haemoglobin (Hb) as substrate and tyrosine determination, which represents the soluble nitrogen. The new method proposed in this paper seems to be more precise and well fitting for cereal proteinases, as it uses natural substrate, instead of Hb.

MATERIAL AND METHODS

In order to show the specificity of native plant peptidases the determinations were carried out on 60% flours of the varieties: Carola, Grana and Helenka, as well as Hb as substrates and two plant enzyme extracts — germinating wheat grains and the bromelain commercial preparation (Merck). Comparison of the action of these two enzymes should demonstrate the necessity of using standard natural substrate instead of Hb.

Standard substrate preparation. It consists of gluten extract chosen from among three of the above mentioned flours. To obtain it flour was extracted by means of shaking with a 3,5 fold volume of 0,05 M acetic acid, three times for 1 h, following the threefold extraction with 0,01 M pyrophosphate buffer pH 7,0 (to remove albumins and globulins). The native peptidases present in the extract were inactivated by heating for 10 min. in a boiling water bath. Then the extract was standardised to a protein content of 9 mg/ml and as standard substrate could be stored in cold for one week.

The rates of digestion of wheat proteins and Hb were investigated with the use of bromelain of 1 mg/ml concentration, as well as the germinating wheat extract. The latter was obtained by extraction of wheat grains germinating for 7 days by homogenisation for 4 min. in two volumes of acetate buffer pH 3,8. This homogenate was centrifuged at 8000 rpm during 10 min. and dialysed against the same buffer, to which some cysteine was added. The dialysate was then concentrated four times in the same tube with powdered sucrose.

The incubated sample contained 1 ml substrate protein (9 mg) and 1 ml of enzyme: bromelain (1 mg) or germinating grain extract (4,5 mg protein). Incubation was performed at 37°C for 30 min. when bromelain was used and 60 min. in case of wheat peptidase. The enzyme was inactivated with the trichloroacetic acid (TCA) of final concentration 3,2%, and the precipitated protein was centrifuged at 16 000 rpm for 15 min. The contents of hydrolysis products were determined in the supernatant

by the method of Reifer and Tarnowska (1951) and that of Anson (1963).

The most suitable substrate, as concerns the proteolysis rate was used for comparative determinations of proteolytic activity of some wheat flours, according to the method described below.

RESULTS AND DISCUSSION

It is known, that cereal peptidases are rather specific to proteins contained in grain, therefore the use of Hb as standard substrate does not seem correct. For activity determination the application of standardised gluten solution seems more suitable. However, it was demonstrated earlier (Liss, Kączkowski, 1975), that gluteins isolated from flours of various baking quality exhibit different accessibility of the peptide bonds, and so — a different rate of proteolysis. This property can be illustrated by the results given in Table 1, which concerns three gluten samples and Hb, as well as two methods of proteolysis product determination.

Table 1

Liberated npN by native wheat peptidases and bromelain determined by two methods
($\mu\text{g}/\text{hour}/\text{mg}$ protein)

Sample of protein	Native wheat peptidases			bromelain		
	tyr. N	total N	N total/ N tyr.	tyr. N	total N	N total/N tyr.
Carola	1.90	38.9	20.5	4.06	157.7	38.8
Grana	1.68	34.1	20.3	4.08	159.8	39.2
Helenka	1.84	36.1	19.7	4.94	186.5	37.8
Hb	2.22	44.1	19.9	3.52	120.9	34.4

It is seen from Table 1, that differences in the digestion rates of wheat gluten samples amount to 15%, but in the case of samples more differentiated in quality, they may be even higher (Liss, Kączkowski, 1975). Therefore application of standard substrate for determination of proteolytic activity seems necessary. In this case the substrate most accessible for proteolysis should be most favourable — in these experiments, the protein extracted from Carola flour. For total comparability of results, concerning proteolytic activity, the rate of substrate proteolysis should be related to that of Hb decomposition. It is proposed arbitrarily to use the standard substrate, for which the ratio of proteolysis rate is $S/\text{Hb} = 0.88$, as determined by Reifer's method (Reifer, Tarnowska, 1951). The substrate standardised in this manner can be

stored in the form of flour in the tightly closed vessel under nitrogen and at $0^{\circ} - 4^{\circ}\text{C}$ for at least one year without any change of proteolysis rate.

If the results concerning the proteolysis product determined by the method of Anson (1963) and those of Reifer and Tarnowska (1951) based on acid combustion are compared, it can be concluded, that both are rather strictly correlated each other in case of action of native peptidases. In this case the variations of the ratio N total/N tyrosine do not exceed 4% for different protein samples used. This may indicate, that native peptidases express the different substrate specificity of separate enzymes in the mixture, which causes the generally nonspecific liberation of proteolysis products from differentiated gluten proteins, as well as Hb. This is in agreement with the well known heterogeneity of peptidases extracted from wheat. The composition of proteolytic enzymes, however, in various wheat samples may be different, therefore, the use as substrate of a mixture of wheat proteins should be more adequate in view of their changable specificity. However, when bromelain was used for proteolysis, the results were slightly different. The ratio total N/total tyrosine mentioned above, was in this case about two times higher. This may have been connected with the relatively slow liberation of tyrosine in the case of bromelain action. Moreover, this ratio in the case of Hb differed by about 10%, as compared to gluten proteins. It is also of interest, that Hb was digested when bromelain was used about 15% slower, than gluten proteins, whereas the relation was reversed, when native peptidases were added. It should be rather expected, that gluten should be digested much faster than Hb, when native peptidase was used, as they both (enzyme and substrate) are of the same origin. It would seem, that Hb represents more denaturated protein, and its preparation might not always lead to the same denaturation stage.

On the basis of the results in table 1 the application of Reifer and Tarnowska's (1951) method, or any other based on acid combustion, should be recommended in the case of the determination of proteolysis products of wheat protein, since they give more precise results. However, for serial analyses, particularly, when proteolysis products are determined with the use of wheat peptidases (for example determination of proteolytic activity) Anson's simpler method (1963) can also be applied. In this case however, the concentration of NaOH should be increased to 1 M because of the higher final concentration of TCA than that prescribed in Anson's original method.

Determination procedure. The proteolytic activity of flour or ground grain should be determined as follows. 1 g of material is extracted with 3,5 ml 0,05 M acetic acid by 1 h shaking and centrifuged at 16 000 rpm for 10 min. In the supernatant the concentration of protein is determined and adjusted to 5 mg/ml. For incubation 5 ml of substrate solution

(containing 45 mg of protein) and 1 ml of flour extract are pipetted and 1 drop of toluol added to avoid microbial infection. Parallely and in an analogous way the control sample is prepared, in which the enzyme is inactivated by heating the extract 10 min. in a boiling water bath. Both samples are incubated at $37^{\circ}\text{C} \pm 1^{\circ}$ for 96 hours in a thermostat. After incubation, proteins are precipitated in both the proper and the control sample, with TCA of 6% final concentration and after 30 min. storage in cold ($0-4^{\circ}\text{C}$) centrifuged off at 16 000 rpm for 10 min. In both supernatants the liberated nonprotein N (npN) is determined as above. The measure of activity is the amount of npN in milligrammes, which is liberated during 96 hours by the enzyme contained in 1 g of dry matter in the proper sample, when the npN liberated in the control one is subtracted. The proteolytic activity (PA) should be calculated therefore, as follows:

$$\text{PA} = \frac{\text{P}}{5} \times (\text{npN}_p - \text{npN}_c)$$

where: P — whole protein contained in 1 g of material
 npN_p — npN liberated in proper sample (in mg)
 npN_c — npN liberated in control sample

Table 2

Proteolytic activity of some wheat samples harvested in 1975 (mg of npN/g of dry matter of flour)

Sample	parallels		average	% difference
Nadzieja	2.43	2.54	2.49	4.41
Grana	2.90	2.94	2.92	1.37
Mironowskaja 808	2.18	2.08	2.13	4.19
Bezostaja 1B	1.86	1.86	1.86	0.0
Prinepi	3.19	3.19	3.19	0.0
Palomares	2.07	1.96	2.02	5.44
Manella	6.80	6.73	6.77	1.03
Splendeur	3.01	3.01	3.01	0.0
Somme	7.31	7.57	7.44	3.49
Luna	4.01	4.01	4.01	0.0
Ursynów	6.93	6.90	6.91	0.43
Norin 10 × Brevor	5.25	5.42	5.34	3.18

By the described method the proteolytic activity of 60% wheat flours was determined. The results given in Table 2, each being in two parallels show, that the activities differ from one another, but the error in the case of particular parallel samples was observed to be not higher than 5%.

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Oznaczanie aktywności proteolitycznej w zbożach

STRESZCZENIE

Przedstawiono wyniki wskazujące na zróżnicowane działanie rodzimych enzymów proteolitycznych pszenicy i bromelainy na białko pszenicy różnej jakości, jak również nie specyficzne działanie tych enzymów na hemoglobinę. Dlatego uznano za celowe opracować nową metodę oznaczania ich aktywności, polegającą na użyciu standardowego, naturalnego substratu, który stanowi wyciąg białek glutenowych z określonej mąki. Podano sposób doboru substratu standardowego, tok oznaczania aktywności, a także przykłady oznaczeń z zastosowaniem opisanej metody dla pewnej liczby prób pszenicy.