The Proteolytic Enzymes of Barley and Malt. I. Extraction of Peptidyl Peptide Hydrolases (Endopeptidases) with Activity at pH 5 from Malt

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ABSTRACT

As a first step in research on the properties of proteolytic enzymes of barley and malt, the extraction from malt of peptidyl peptide hydrolases with activity at pH 5 was studied. The effect of various factors such as pH, temperature, and additions to the extractant was investigated. The extraction of water-soluble proteases is optimal after some minutes at room temperature. Addition of cysteine and nylon 6.6 to the extractant increases the yield. The extraction of proteases soluble in 8% NaCl solution is completed after an extraction time of approximately 30 min. at 40°C., provided the extractant is buffered to pH 5. The proteolytic activity in the extracts is most stable at pH 5.

It is well known that the proteolytic activity of barley and malt is important in malting and brewing, but little is known about the nature of the proteolytic enzymes, probably because of various difficulties in this area of research. A major difficulty arises because only a very small part of the complicated mixture of proteins found in barley and malt possesses proteolytic activity. However, the rapid development of methods for protein separation during recent years has considerably increased the chance of success.

Our research was confined to the peptidyl peptide hydrolases (EC group 3.4.4, hereafter referred to as PPH), according to the classification of Bergmann, endopeptidases. The purposes of our study were to find how many enzymes with PPH activity are present in barley and malt, and what their specific demands are with respect to the amino acids adjacent to the split peptide linkage.

In 1902 Weis (1) postulated that at least two proteases are present in malt; this was supported by Kringstad *et al.* (2, 3). During our investigation a publication by Enari *et al.* (4) on the same subject appeared; they found at least five proteolytic enzymes in barley and malt, by means of column chromatography (ion-exchange and molecular-sieve) and electrophoresis.

It is essential for our purpose that all PPH's be extracted as completely as possible so that enzymes which are soluble with difficulty are not overlooked. We used current methods for extraction of albumins and globulins. However, we judged the effectiveness of the extractions not by the quantity of extracted protein, but by the quantity of extracted PPH.

As malt possesses a higher proteolytic activity than barley the present research was performed with malt.

MATERIALS AND METHODS

Malt

Malt of the barley variety Proctor (harvest 1961) was germinated normally in a brewery (Verenigde Nederlandse Brouwerijen d'Oranjeboom

N.V.). It was dried in a stream of air at 30°C. to a water content of 9%.

Determination of PPH Activity

The most suitable test was one based on the decrease of viscosity of a gelatin solution by proteolytic hydrolysis, because it is sensitive and simple. The following procedure was evolved: 1 g. of gelatin ("Gelatine voor bacteriologische doeleinden," Lijm- en Gelatinefabriek, Delft) is stirred at 40°C. for 10 min. with 10 ml. 0.02*N* acetate buffer, pH 5. Then 3 ml. of the PPH solution is added. After a short mixing time, the mixture is transferred to an Ubbelohde viscometer prewarmed at 40°C., and the decrease in viscosity is measured at regular intervals. The only objection to this assay is the impossibility of using a unit of activity which is in accordance with the recommendations of the International Union of Biochemistry. We use a unit that fits in directly with our assay. Apart from a sharp decrease in viscosity during the first minutes of the determination, which also occurs in the blank, the decrease in viscosity was found to proceed linearly, provided the enzyme activity was not too high. The slope of the line, viscosity *vs.* time, is proportional to the PPH activity. We defined one unit of PPH activity as that quantity of PPH which gives a viscosity decrease of 0.2 cp. per hr. Since the decrease of viscosity of a solution of gelatin by PPH is different for each batch of gelatin, we always used gelatin from one stock for comparable experiments. The described method is very similar to that used by Enari *et al.* (4).

In some cases the activity was measured with the method described by Sandegren and Klang (5). The substrate is hemoglobin; the activity of the enzyme is found by spectrophotometric determination of the amount of free aromatic amino acids after precipitation of proteins and peptides by trichloroacetic acid. This method was not sensitive enough for the majority of our experiments. Unless otherwise stated, the viscosity method was used. With most enzyme samples the decrease in viscosity was followed for 45 min.; samples with low activity were incubated for a longer time, after which the difference in viscosity, as compared with a blank, was determined.

Determination of Quantity of Anthocyanogens in Malt Extract

This was performed by the method of McFarlane (6).

RESULTS OF EXPERIMENTS

Extraction of Albumin-PPH

Malt was milled with a Glen Creston laboratory sample mill. The albumin-PPH were extracted with distilled water. The effect of the following factors on the extraction was investigated: milling time and fineness of the meal, extraction of fats from the meal before protein extraction, extraction temperature, extraction time, ratio extractant:meal, additions to the extractant.

Effect of Milling Time and Fineness of Meal. Coarsely milled malt was ground further with a vibrating ball mill; samples were taken during grinding, in which the proteolytic activity was determined by the method of Sandegren and Klang. We divided the sample with the highest activity in fractions of different fineness by sieving. In each of these fractions the proteolytic activity was determined also.

It appeared that grinding in the vibrating ball mill reduces the extractable

proteolytic activity. The addition of solid carbon dioxide in the course of milling increased activity only slightly, indicating that heat was not denaturating the enzyme; probably there was mechanical denaturation.

From the composition and the proteolytic activity of the various fractions after sieving (Table I), we concluded that the proteolytic enzymes occur particularly in the aleurone layer and in the germ of the malt grain. This is in accordance with both the results of a study by Engel and Heins (7) and the following observations. Malt grains were divided into germ, endosperm, aleurone, and bran; the proteolytic activity per g. was determined. The highest activity was found in the germ; the activity in the aleurone layer was about one-fifth of that in the germ; the proteolytic activity in the endosperm and in the bran was nearly nil.

TABLE I

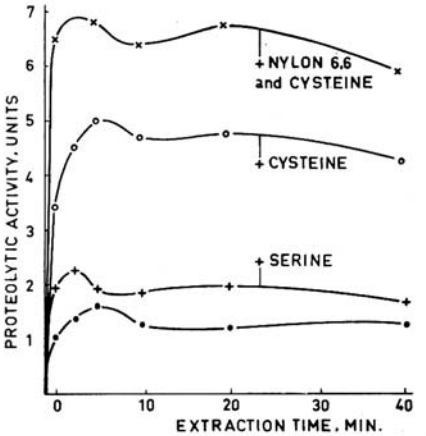
PROPERTIES OF MALT FRACTIONS OBTAINED BY SIEVING

	Mesh Size of Particles					
	< 10	10–20	20–30	30–40	40–50	> 50
Weight of fraction, g.	2.8	18.3	15.3	5.3	6.8	11.7
Proteolytic activity ($E \frac{1\text{ cm.}}{263\text{ nm}}$ per 100 mg.)	0.09	0.32	0.53	0.41	0.19	0.15
Composition of fraction	Bran	Bran, brown flour	Bran, brown flour	Brown flour	Yellow flour	White flour

Extraction of Lipophilic Compounds from Malt. To avoid possible interference with the experiments, lipophilic compounds occurring in malt meal were extracted with cold acetone (–20°C.). The effect of this extraction on the proteolytic activity of the meal, as tested by the method of Sandegren and Klang (5), appeared to be negligible.

Effect of Extraction Time and of Addition of Reducing and Tannin-Binding Compounds. Malt meal, from which particles over 10-mesh (bran) were discarded, was stirred with distilled water at 0°C. (ratio water:meal, 4:1). During this extraction, samples were taken from the water-meal suspension. These were centrifuged for 2 min. at 22,000 × *g*. In the supernatants the PPH activity was determined (curve at bottom of Fig. 1). The PPH activity in the extract arrives at an optimum within 3 to 5 min.; after that the loss of activity over some minutes surpasses the extraction of enzyme. Possible cause of the activity loss may be oxidation of –SH groups in the enzyme or binding of the enzyme by tannins.

To test the influence of oxidation during extraction, we extracted samples of meal with several concentrations of cysteine in water. From Fig. 2 it appears that addition of cysteine to the extractant augmented considerably the PPH activity in the extract. Addition of glutathione, mercaptoethanol, or mixtures of the various compounds gave the same result. That the higher activity in the extract is not a consequence of the extraction of globulin-PPH as a result of the addition of electrolyte to the extractant appears, first, from the fact that the nonelectrolyte mercaptoethanol has a similar effect, and sec-



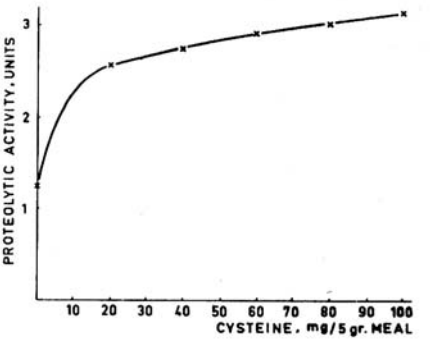


Fig. 1 (left). Effect of additions to extractant on extraction of albumin-PPH from malt.

Fig. 2 (right). Effect of cysteine in extractant on extraction of albumin-PPH from malt.

ond, from the fact that an equal quantity of serine does not affect the extraction appreciably (Fig. 1, curve "+ serine").

In malt extract, protein-tannin complexes do occur (8). The addition of tannin-binding compounds might therefore enhance the protein solubility and, as a consequence, the PPH activity in the extract. As tannin-binding compounds the insoluble polymers nylon 6.6 and agent AT 496 (a polyvinylpyrrolidone) were used. Mixtures of malt meal with different quantities of these compounds were extracted with 0.25% solutions of cysteine. The suspensions were centrifuged; the PPH activity and the anthocyanogen content was determined in the supernatants. The results (Fig. 1, curve "+

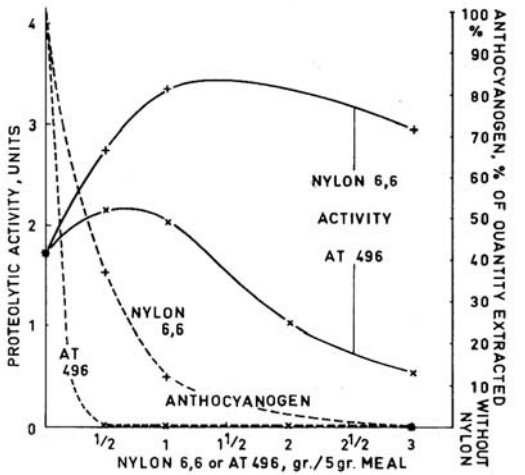


Fig. 3. Effect of nylon 6.6 or AT 496 in extractant on albumin-PPH activity and anthocyanogen content of extract.

nylon 6.6 and cysteine" and Fig. 3) indicate a favorable effect of nylon 6.6 on the extraction; agent AT 496 is less effective.

Effect of Extraction Temperature, Water:Meal Ratio, and Method of Stirring on Extraction of PPH. Malt meal mixed with nylon 6.6 (5:1, w./w.) was extracted with 0.25% solution of cysteine in distilled water for 10 min. The temperature, the water:meal ratio, and the method of stirring were varied in different experiments. After the extractions, the suspensions were centrifuged for 2 min. at 22,000 $\times$ *g;* the PPH activity was determined in the supernatant.

The effect of the extraction temperature is shown in Fig. 4 (curve "albumin-PPH"). For the extraction of albumin-PPH, about 30°C. is most favorable.

We did not find an effect of the ratio meal:water on the extracted PPH activity per g. meal, provided the ratio was over 2. With 2 parts extractant to 1 part meal, the extraction yield decreased. It was not of importance to this work; either this results from a kind of saturation of the extractant, or it is connected with the different mixing properties of this very viscous mixture.

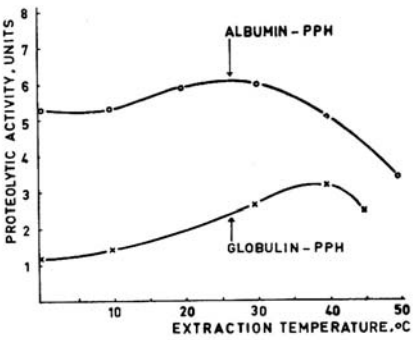


Fig. 4 (left). Effect of extraction temperature on extraction of PPH.

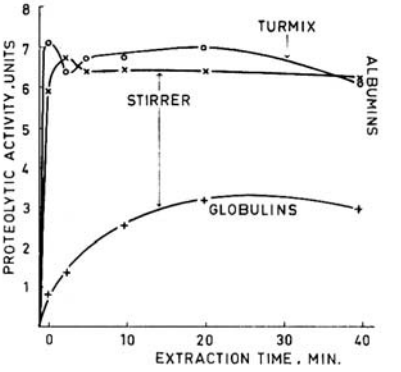


Fig. 5 (right). Effect of extraction time and method of stirring on PPH activity in extract.

Two methods of stirring were tested; a mechanical stirrer (1,000 r.p.m.), and a mixer (7,000 r.p.m.).

A higher stirring speed appeared favorable, until the heat development began to be harmful (after about 30 min.) (Fig. 5).

Extraction of Globulin-PPH

The malt meal used had been extracted with a 0.25% solution of cysteine in distilled water twice for 10 min. and once for 30 min., to discard the albumins.

Padmoyo *et al.* (9), studying the extraction of globulins from various cereals, found that with 8% NaCl solution a complete extraction is obtained. We assumed this to be true also for the extraction of globulins from malt.

Effect of pH of Extractant on Extraction of Globulin-PPH. The material

was extracted at room temperature with buffer solutions (containing 8% NaCl) which differed in pH value. After adjustment of the pH of the extracts to 5, the PPH activity in the extracts was determined. It is shown by the results that the best pH for the extraction is about 5 (Fig. 6). In the follow-

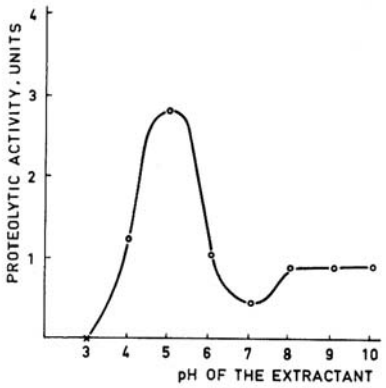


Fig. 6. Effect of pH of extractant on extraction of globulin-PPH.

ing experiments, globulin-PPH was extracted with a solution of 8% NaCl in 0.1*M* acetate buffer, pH 5.

Effect of Time and Temperature of Extraction. Globulin-PPH was extracted at different temperatures. During the experiment at 25°C., samples were taken after different times. All were centrifuged and the PPH activity was determined in the supernatants. The optimum temperature for extraction was 40°C. (Fig. 4, "globulin PPH").

An extraction time of 30 min. was found to be optimal (Fig. 5, "globulins").

Effect of Cysteine on Extraction. These experiments were performed with malt meal from which the albumin-PPH had been extracted—without the use of cysteine, however, to avoid a possible effect of cysteine remaining from the albumin extraction. It appeared that cysteine in the extractant gives the globulin-PPH in a yield about 50% higher.

Efficiency of Extractions

Finally we investigated whether the PPH activity is extracted completely with the evolved procedure. For this purpose a sample of meal was extracted three times under the conditions which were found most favorable; the PPH activity was determined in the extracts. The centrifuged meal with the accompanying extractant was weighed after each extraction. From these data the theoretical value of the PPH activity in the second and third extracts was calculated, starting from the assumption that the first extraction had been complete. Both with the albumin extraction and with the globulin extraction it was found that the second and third extractions provided even less activity than was to be expected, if all PPH activity was extracted during the first extraction. This might be due to denaturation of PPH during the pro-

cedures, and to the fact that the extractant trapped in the meal is partly inaccessible to proteins (10).

Stability of PPH Activity in Malt and in Malt Extracts

For further experiments it was necessary to know at which temperature and pH the malt PPH is most stable.

The effect of pH on the enzyme stability was tested for both albumin-PPH and globulin-PPH. In both cases equal parts of one enzyme preparation were brought to different pH values (4, 5, 6, and 7) with diluted HCl or diluted NaOH. The samples were diluted to the same volume and stored. The PPH activity was followed during storage. The favorable storage pH values for albumin are 4 and 5 (Fig. 7). Globulin-PPH seems relatively stable at pH 5 for a longer time (Fig. 8).

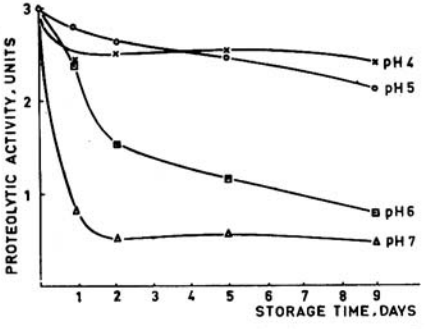
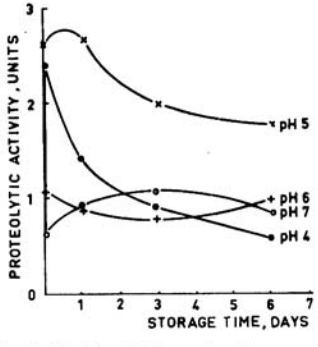


Fig. 7. Effect of pH on stability of albumin-PPH from malt.

The effect of temperature on stability was tested with a mixture of albumin- and globulin-PPH in solution buffered at pH 5. The solution was heated at the test temperature; each 10 min. a sample was drawn and cooled



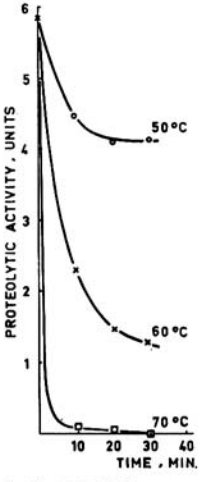


Fig. 8 (left). Effect of pH on stability of globulin-PPH from malt.
Fig. 9 (right). Loss of activity of PPH from malt by heating at pH 5.

as soon as possible to 0°C. The activities of the samples are shown in Fig. 9. It appears that at 70°C. the activity is lost within 10 min. At 50°C. a part of the activity is lost in 10 min.; the remainder seems more stable. This might be a further indication of the presence of different PPH's in the extract, one stable at 50°C. and pH 5 and one unstable under the same conditions.

Dry preparations, such as malt meal, lyophilized extract, or malt proteins precipitated with acetone, do not lose activity at room temperature over a number of days. This is not illustrated with data.

DISCUSSION

The results show that PPH activity occurs in both malt albumins and malt globulins much higher in the first than in the latter.

Higher PPH activity is found in both fractions after extraction in the presence of cysteine; hence it is possible either that we extract a –SH enzyme, or that cysteine enhances the solubility by disrupting S–S linkages.

The PPH activity can also be increased by addition of a tannin-binding compound to the extractant. This decreased the anthocyanogen content in the extract. However, as shown by Fig. 3, this correlation is not complete, for whereas AT 496 removes more anthocyanogen, the effect on the PPH extraction is less than with nylon 6.6. Probably the latter compound binds more nonanthocyanogen polyphenols which likewise inactivate PPH. The addition of cysteine and nylon 6.6 to the solvent for the extraction of albumin-PPH does not prevent the activity drop in the curve "PPH activity *vs.* extraction time" between 5 and 10 min. extraction time (Fig. 1). Another explanation for this drop in activity is the presence in the malt of an enzyme inhibitor which was extracted more slowly than the enzyme.

The PPH of the albumin fraction can be extracted much faster than the globulin-PPH (Fig. 5). Probably the former has lower molecular weight.

We found an optimum for the extraction of globulin-PPH from malt at pH 4–5; Enari *et al.* (11) found an optimum for the extraction of globulins at pH 7. Probably this difference is due to the optimum of stability of the PPH, which is about pH 5 (Figs. 7 and 8).

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Literature Cited

1. Weis, F. Thesis, Copenhagen University, 1902.
2. Kringstad, H., and Olsen, J. Ueber die proteolytischen Enzyme von Gerste und Malz und die Hitzestabilität der proteolytischen Malzenzyme. European Brewery Conv., Proc. Congr. 5th, Baden-Baden, 1955, pp. 318–323.
3. Kringstad, H., and Kilhovd, J. Weitere Studien über die proteolytischen Enzyme von Gerste und Malz. European Brewery Conv., Proc. Congr. 6th, Copenhagen, 1957, pp. 67–71.
4. Enari, T-M., Puputti, E., and Mikola, J. Fractionation of the proteolytic enzymes of barley and malt. European Brewery Conv., Proc. Congr. 9th, Brussels, 1963, 37–44.
5. Sandegren, E., and Klang, N. On barley-amylase and -proteinase. J. Inst. Brewing 56: 313–318 (1950).

6. MCFARLANE, W. D. Determination of anthocyanogens. J. Inst. Brewing 67: 502–506 (1961).
7. ENGEL, C., and HEINS, J. The distribution of enzymes in resting cereals. Biochim. Biophys. Acta 1: 190–196 (1947).
8. CHOLLOT, B., CHAPON, L., and URION, E. Précurseurs polyphénoliques et protéiques du trouble de oxydation peroxydasique. European Brewery Conv., Proc. Congr. 8th, Vienna, 1961, pp. 334–349.
9. PADMOYO, M., and HÖGL, O. Beiträge zu der Papierelectrophorese des Getreide-Eiweisses. Mitt. Gebiete Lebensm. Hyg. 53: 290–319 (1963).
10. MEREDITH, O. B., and WREN, J. J. Determination of molecular-weight distribution in wheat-flour proteins by extraction and gel filtration in a dissociating medium. Cereal Chem. 43: 169–186 (1966).
11. ENARI, T-M., NUMMI, M., MIKOLA, J., and MÄKINEN, V. Extraction of albumins and globulins of barley grain. Finska Kemistsam fundets. Medd. 71: 44–52 (1952).

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