Proteolytic Activity Based on a Malt Flour Substrate Interaction

PAUL R. WITT, Jr., and **EDWARD A. TOUSIGNANT,** Northwestern Malt and Grain Company, Minneapolis, Minnesota

ABSTRACT

It has been established that malt contains enzymes which exist in the "bound" state and which are believed to be active in the presence of a suitable substrate. The present data reveal that the rate of casein digestion by whole (ground) malt is equaled by a comparable equivalent of filtrate from malt extracted with 5% K_2SO_4 containing versenate and cysteine (adjusted to pH 5.5). Dosage-response studies showed reaction rates which are linear through 15- and 30-min. periods of digestion. Extensive investigation was carried out at a reaction temperature of 40°C. Activity was higher at 50°C. and was optimum at pH 6.0. Reaction was retarded at pH 5.0 and 6.7. The relation of enzyme to substrate at pH 6.0 reveals that optimum substrate level is between 0.5 and 1.0% casein. Proteolytic values for a series of barley malts are presented. The recorded variation in proteolytic activity reflects the processing history of the malts.

The purpose of this study was to develop a method of estimating the total or near-total proteolytic activity of malt.

Previously reported procedures utilize extracts of malt or use ground malt directly, and employ casein (1,2), gelatin (3), edestin (4), or hemoglobin (5,6) as substrate.

Measurement of solubilized nitrogen within an autolytic digestion has been advocated (7,8), and another method suggests the use of heat-inactivated barley flour as substrate (9).

Gelatin viscosity measurements generally require enzyme in solution. While this is not questioned as to efficacy, a procedure employing a large enzyme-to-substrate ratio is suspect; Massart (3) employs the equivalent of 1.5 g. of malt in 22.5 ml. of gelatin-containing medium.

A relatively large quantity of malt is also used in a method reported for hemoglobin (5), where 5.0-g. portions of malt are digested with 2.25 g. hemoglobin in 50 ml. of buffered medium.

Autolytic methods generally use a heavy malt suspension, which may be interpretive for nitrogen solubilization within a brewer's mash. However, these methods give little attention to enzyme kinetics.

Under one well-known procedure (10), after autolytic digestion for 1 hr., soluble nitrogen is determined as the blank, and actual proteolytic activity is estimated by subtracting the blank from the quantity of soluble nitrogen formed in 3-hr. digestion of the same malt.

A procedure specifically designed to compare malts intended for brewing (8) determines the amount of soluble nitrogen after 2-hr. mashing at 40° and 60°C.; a blank is mashed and filtered at 4°C.; and this "preformed" nitrogen is subtracted from that developed at the higher temperatures.

The present procedure measures the amount of nitrogen (as tyrosine) which is solubilized from casein by a whole-malt flour suspended in a cysteine-

versenate buffer. The rate and quantity so observed are compared to that solubilized by an infusion filtrate with several aqueous extractants.

Evidence of bound enzymes in malt has been demonstrated by Witt and Ohle (11). Kringstad and Kilhovd (12) recently reported an estimate of the relative activity of bound and soluble proteases in malt.

No clear-cut opinion has been advanced to explain the condition of the so-called bound protease or the mechanics of its reaction. It is reasonable to assume that some of the proteinaceous material of the malt is denatured during the ventilation, heating, and desiccation that occur during kiln-drying. Moreover, it is probable that the bound enzyme could exist in a partially denatured state.

Boyer *et al.* (13) demonstrated that approximately 0.45*M* sodium sulfate negated the denaturing effect of 2.5*M* guanidinehydrochloride on 2% serum albumin. It is postulated that the sulfate ion exercises this protective effect.

As the present study developed, proteolytic activity was observed in aqueous extracts as well as in those resulting from 0.5% sodium chloride and a 5% potassium sulfate extraction. Use of sodium chloride would have been expedient, as a common filtrate could have been used to determine both amylolytic and proteolytic activity. The 5% potassium sulfate was selected because it is approximately the molar equivalent of the 0.45*M* sodium sulfate used by Boyer.

MATERIALS AND METHODS

Substrate and Extractants

The procedure of Weissler and Garza (1) was used (a) to select the casein, solubilize it, and adjust its pH, and (b) to prepare the ("regular") cysteine-versenate-phosphate buffer.

Sodium chloride: Dissolve 0.5 g. of the C.P. salt in 100 ml. distilled water.

Potassium sulfate: (a) Dissolve 5.0 g. of the C.P. salt in 100 ml. distilled water. (b) Where additions are used, dissolve 0.3 g. cysteine hydrochloride and 0.7 g. of the versenate in 5% potassium sulfate. Adjust reaction to pH 6.0 with dilute sodium hydroxide or dilute sulfuric acid.

Ninhydrin (triketo hydrindene hydrate) and Methyl Cellosolve (ethylene glycol mono methyl ether), obtained from Fisher Scientific Co.

Types of Malt

All malts were prepared from the same lot of 1964 crop Larker barley. Other than indicated, steeping and germinating periods were the same. Processing procedures were as follows:

R 1141–R 1143. One lot of Larker green malt germinated 5 days (60°–62°F.) was kilned to 7–8% moisture at 120°F.; additional 4-hr. drying: R 1141 at 150°F.; R 1142 at 180°F.; R 1143 at 195°F.

R 1178A. Larker, germinated at 60°–62°F. for 6 days and dried successively at 140°, 160°, 170°, and 180°F.

R 1194A. Germinated at 70°–72°F. for 4 days and 60°F. for the 5th day. Kilning as in 1178A.

R 1195A. Germinated at 50°–52°F. for 5 days. Kilning as in 1178A.

R 1176A. Germinated at 60°–62°F. for 4 days only. Kilning as in 1178A.

All malts in the R series were lightly kiln-sulfured. The distillers' malt was given a greater amount of sulfuring.

METHODS

As a standard for developing methodology, one uniform lot of brewers' malt was used. A minimum of 25 g. was freshly ground through the 1.0-mm. screen of the Wiley mill and mixed thoroughly on a glazed surface before samples were taken.

Procedure

Place 50- and 100-mg. samples in 25 × 150-mm. test tubes and extract for 5 min. at 40°C. in 2.0 ml. of regular cysteine-versenate-phosphate buffer (pH 6.0).

At zero time, add 5.0 ml. casein (adjusted to pH 6.0 unless otherwise specified) at 40°C. to the tubes containing the malt suspension. After 15-, 30-, 45-, and 60-min. digestion periods, add 3.0 ml. of trichloroacetic acid (TCA).

Permit the appropriately timed blanks to digest for the designated periods of time, arresting autoproteolysis with 3.0 ml. of TCA, followed by 5.0 ml. of casein solution.

Filter and read absorbance against the appropriate blank in a 10-mm. cuvet at 280 mμ. (The soluble nitrogen formation can also be estimated with ninhydrin; the procedure is given below.)

Dilute 2.5 ml. TCA filtrate to about 25 ml. with distilled water and adjust to pH 6.0 with NaOH. Make to final volume of 50 ml.

Place 1.0 ml. of the diluted filtrate and 2.0 ml. ninhydrin-citrate buffer mixture, as recommended by Fels and Veatch (14), in a 14 × 150-ml. test tube, cover with aluminum foil, and heat for 20 min. in a vigorously boiling water bath. Cool immediately, dilute to 10 ml. with a 50-50 mixture of ethanol (or 1-propanol) and water, and mix by inversion. Transfer to a cuvet and read against an appropriately reacted digestion blank at 570 mμ.

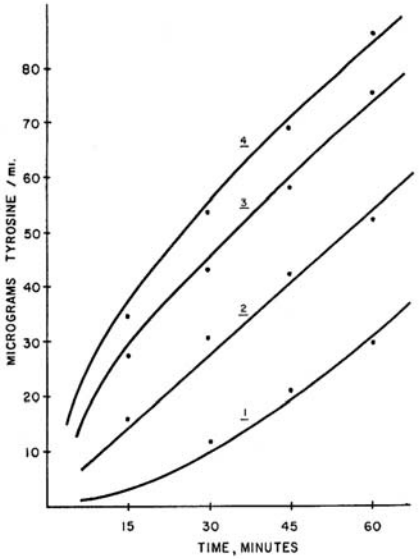
Determine developed nitrogen from a standard ammonium sulfate curve as directed by Fels and Veatch (14).

When tyrosine formation, as determined directly on the TCA filtrate (at 280 mμ), was compared with that developed by ninhydrin, curves were parallel.

RESULTS AND DISCUSSION

Enhancing Effect of Versenate and Cysteine

Figure 1 shows the activity of the finely ground malt (extracted 1 hr., 20°C., with 0.5% NaCl and with 5% K_2SO_4) and the casein (digested at 40°C., pH 6.0, with the filtrate equivalents to 100 mg. malt), as well as the increased tyrosine formation due to enhancement of the filtrate with versenate-cysteine buffer (added to the filtrate immediately before casein digestion). Curve 1 implies activation of the enzyme during incubation or substrate digestion. A comparable but less pronounced tendency seems to be reflected in curve 2. Curve 3 exemplifies the greater extraction ability of 5% K_2SO_4. Curve 4 illustrates the full activity due to —SH donor and to versenate protection.



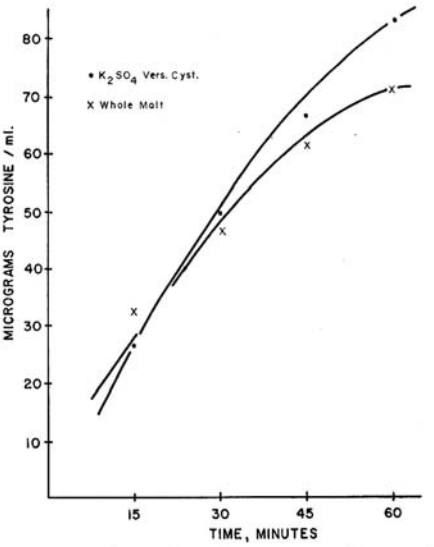


Fig. 1 (left). Enhancing effect of versenate and cysteine during proteolysis of casein by malt enzyme, and comparison of sodium chloride and potassium sulfate as extractants (100 mg. equiv. 10% malt): curve 1, in 0.5% NaCl; curve 2, in 0.5% NaCl casein digest with vers.-cyst.; curve 3, in 5% K_2SO_4; curve 4, in 5% K_2SO_4 casein digest with vers.-cyst.

Fig. 2 (right). Comparison of proteolysis resulting from 100 mg. whole (ground) malt and from a 100 mg. (malt) equivalent of a 5% K_2SO_4 versenate-cysteine extraction.

Filtrate Equivalent vs. Ground Malt

In a further study, versenate-cysteine was added directly to the 5% K_2SO_4 extractant. Figure 2 compares the activity of this 100 mg. filtrate equivalent with the direct action of 100 mg. of ground (whole) malt (extracted in versenate-cysteine buffer before substrate digestion).

The K_2SO_4 extraction seems to represent a condition in which a more complete steady state exists than in that with the whole malt, where the curve appears to drop off prematurely. This may be related to incomplete solubilization of enzyme or incomplete contact with substrate. The purpose of the comparison was to show that, within the 15- and 30-min. digestion periods, the filtrate equivalent demonstrated a comparable rate of activity.

To establish a rate relationship, a 10% malt suspension was extracted with 5% K_2SO_4 (versenate-cysteine) at pH 5.5. The standard inoculum of 2.0 ml. of this filtrate represents 200 mg. malt equivalent. This was further diluted with K_2SO_4 extractant to the following concentrations of malt equivalent: 100, 50, 25, and 12.5 mg.

The dosage (enzyme)-product relationship at 40°C. (pH 6.0) is illustrated in Fig. 3. The 200 mg. equivalent is associated with rapid depletion of the 1% casein substrate. Dilution of this level results in a series of curves with apparent linearity between the 15- and 30-min. intervals.

Increasing quantities of malt equivalent are plotted directly against tyrosine formation in Fig. 4. The straight line after 15 min. of digestion

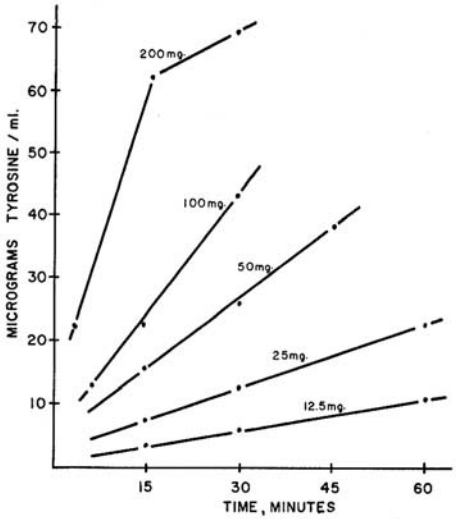


Fig. 3. The digestion of casein at 40°C. (pH 6.0) by incremental quantities of malt equivalent (versenate-cysteine K_2SO_4 extraction).

permits definition of an arbitrary activity unit: $v = kE$; 1.0 unit (E) is equivalent to 10 γ tyrosine/15 min.

To provide further insight into the kinetics of reaction, a 100 mg. filtrate equivalent was reacted with 1% casein (pH 6.0) at 30°, 40°, and 50°C. Figure 5 indicates an increase in activity at 50°C.

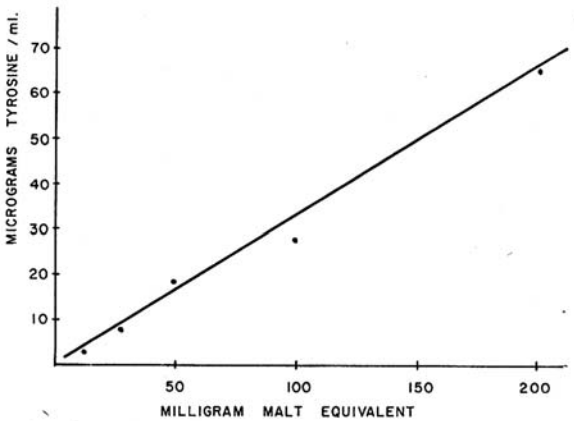


Fig. 4. Tyrosine formation after 15 minutes' digestion resulting from incremental enzyme concentration (1% casein, pH 6.0, 40°C.; versenate-cysteine K_2SO_4 extraction).

Reaction or pH Value of Enzyme Digestion Mixture

To investigate pH value, the reaction of the enzyme-casein medium was adjusted to pH 5.0, as directed in the method cited above (2, Method 68.1).

Since reported pH values of reaction media have ranged from 5.0 to

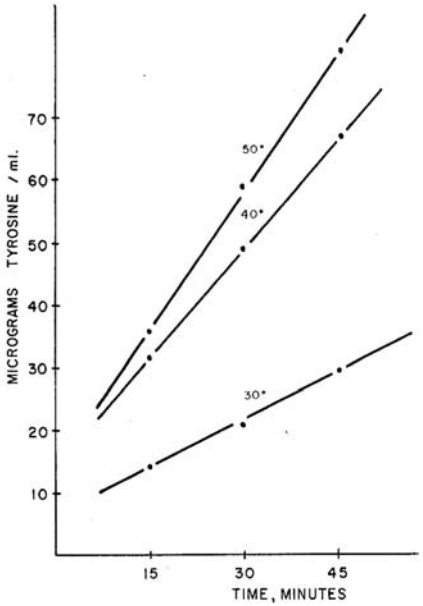


Fig. 5 (left). Digestion of 1% casein (pH 6.0) at 30°, 40°, and 50°C., 100 mg. malt equiv.; versenate-cysteine K_2SO_4 extraction.

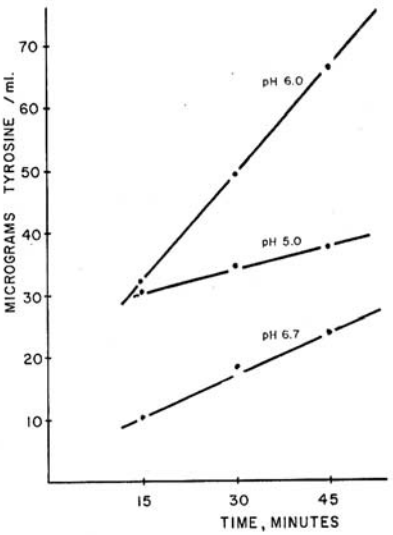
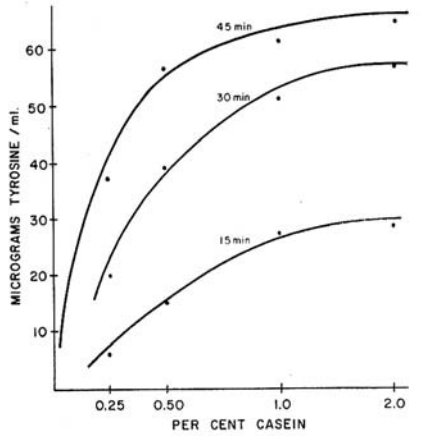


Fig. 6 (right). Digestion of 1% casein (40°C.) at pH 5.0, 6.0 and 6.7; 100 mg. malt equiv.; versenate-cysteine K_2SO_4 extraction.



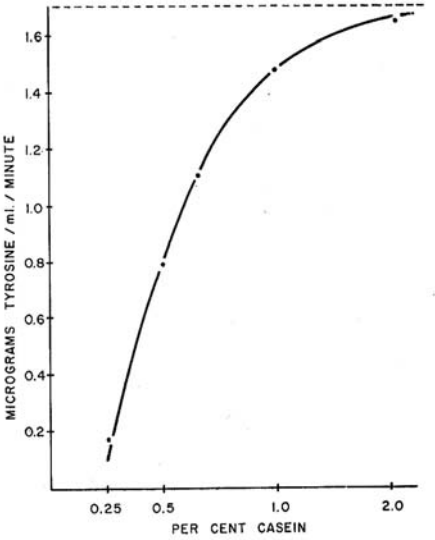


Fig. 7 (left). Effect of increase in substrate concentration on product formation after respective 15-, 30-, and 45-min. periods.

Fig. 8 (right). Effect of increase in substrate concentration on the rate of product formation at 15 min.; 100 mg. malt equivalent.

7.0, a series of experiments was designed to study pH optimum. Figure 6 illustrates the digestion of 1% casein (40°C.) by 100 mg. malt equivalent at pH values of 5.0, 6.0, and 6.7. Formation of tyrosine was approximately the same at pH 5.0 and 6.0 after 15 min.; only a small additional amount was formed within the succeeding 30-min. digestion time. Hammarsten casein was precipitated noticeably below pH 5.5. It is suspected that there is marked depletion of substrate after 15 min. of reaction at 40°C.

Relation of Enzyme to Varying Substrate Concentration

Figure 7 denotes the effect of a 100 mg. malt equivalent (vers.-cyst. 5% K_2SO_4 extraction) acting at 40°C. (pH 6.0) on substrate concentrations of 0.25, 0.5, 1.0, and 2.0%. Curves are plotted at 15-, 30-, and 45-min. digestion periods. Note the leveling-out at about 1% casein concentration.

Figure 8 expresses the same data in terms of rate of tyrosine formation at a 15-min. reaction period.

Activity of Differently Processed Malts

A series of malts were examined for proteolytic activity with the K_2SO_4 extraction procedure.

It has been suggested that one unit of activity results from that quantity of enzyme which forms 10 γ tyrosine per ml. (final TCA filtrate) in 15 min. Since 0.100 g. malt equivalent has reacted in 10 ml. filtrate, we have:

$$\text{Activity in 1.0 g. malt} = \frac{\text{tyrosine/ml./15 min.} \times 100}{10}.$$

The activity of the malts described is shown in Table I, with values for alpha-amylase, diastatic power, and wort-soluble protein. Expression of the latter as a ratio of soluble/total protein is meaningful, because the total protein value of the malts was comparable.

TABLE I

PROTEOLYTIC ACTIVITY OF MALTS PREPARED BY DIFFERENT PROCESSING PROCEDURES

SAMPLE NO.	PROTEOLYTIC ACTIVITY	DIASTATIC POWER	ALPHA-AMYLASE	SOLUBLE/TOTAL PROTEIN
	per g.	*°L.*	*ASBC Units*	*%*
Distillers' malt	370	253	64.0	52.2
R 1141	390	214	51.2	39.9
R 1142	345	164	52.0	42.2
R 1143	185	120	44.3	42.2
R 1178A	300	155	38.4	40.9
R 1194A	300	154	39.4	38.3
R 1195A	260	176	28.6	35.1
R 1176A	165	176	31.0	35.0

Tabulated Data

The manner of processing was so devised as to illustrate characteristics of malt protease.

As has been generally accepted, proteolytic activity is *heat-labile,* as illustrated within 1141, 1142, and 1143 (Table I). A parallel with diastatic power rather than alpha-amylase is noted.

A shortened germinating period (1176A) or a process encouraging under-modification (1195A) tends to reduce proteolytic activity. In this respect, there is no parallel with diastatic power.

A higher germination temperature (1194A) does not reduce proteolytic activity to the degree expected.

Although high enzymatic activity in malt is generally associated with a high level of soluble protein, there exists no direct correlation between the latter and proteolytic activity. It is well known that a high degree of protein solubilization can result from process variation which does not necessarily result in enzyme increase.

Acknowledgment

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Literature Cited

1. WEISSLER, H. E., and GARZA, A. C. Some physical and chemical properties of commercial chillproofing compounds. Am. Soc. Brewing Chemists, Proc. 1965, pp. 225–238.
2. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Cereal laboratory methods (6th ed.), Method 68.1. The Association: St. Paul, Minn. (1957).
3. MASSART, L., J. de Clerck Textbook of Brewing, Vol. 2, p. 363. Chapman Hall: London (1958).
4. LAUFER, S. Studies on the proteolytic activity of barley malt. J. Assoc. Offic. Agr. Chemists 20: 307–330 (1937).
5. ZOCH, L. L., and OLSON, W. J. The influence of barley variety and location grown on the proteolytic activity of malt. Am. Soc. Brewing Chemists, Proc. 1949, pp. 1–5.
6. SANDEGREN, E., and KLANG, N. On barley amylase and proteinase. J. Inst. Brewing 47: 313–318 (1950).
7. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Cereal laboratory methods (7th ed.), Method 22–61. The Association: St. Paul, Minn. (1962).
8. WITT, P. R., JR., and ADAMIC, E. The effect of kiln sulfur dioxide on proteolytic activity during mashing. Am. Soc. Brewing Chemists, Proc. 1957, pp. 37–45.
9. IDOUX, L. Proteolytic activity of malt. Brasserie et Malterie 23: 122 (1933).
10. AYRE, C. A., and ANDERSON, J. A. Varietal differences in barleys and malts. VI. Autolytic proteolytic activity of malt and its correlations with wort nitrogen and barley nitrogen fractions. Can. J. Research 17C: 239–246 (1939).
11. WITT, P. R., and OHLE, R. L. A practical evaluation of laboratory-determined amylolytic data. I. Contrast of methods of analysis with actual alcohol production. Am. Soc. Brewing Chemists, Proc. 1947, pp. 45–50.
12. KRINGSTAD, H., and KILHOVD, J. Weitere studein über die proteolytischen enzyme von Gerste und Malz. European Brewery Conv., 6th, Copenhagen, 1957, p. 67.
13. BOYER, P. D., BALLOU, G. A., and LUCK, J. L. The combination of fatty acids and related compounds with serum albumin. II. Stabilization against urea and guanidine denaturation. J. Biol. Chem. 169: 199–208 (1946).
14. FELS, G., and VEATCH, R. Microdetermination of ammonium and protein nitrogen. Anal. Chem. 31: 451–452 (1959).

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