EFFECT OF MUSHROOM EXTRACT ON THE PHYSICAL PROPERTIES OF DOUGH[1]

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ABSTRACT Cereal Chemistry 53(3): 420-428

Mushroom extract was found to have an oxidative effect on unfermented dough. The effect was due to a nondialyzable fraction of mushroom extract. In extensigraph response, 1.5 g of mushroom dry matter was approximately equivalent to 6 mg of potassium iodate. The oxidative effect of mushroom extract was lost by preincubation of the extract with phenylthiourea or by pretreatment of flour with N-ethylmaleimide. From comparison of the effect of mushroom stipe and pileus, it was shown that pileus extract contains a component other than tyrosinase causing a deleterious effect on dough, independent of SH-groups in flour. Commercial purified tyrosinase caused an oxidative effect similar to that of mushroom stipe extract.

An aqueous extract of edible mushroom was reported by Bessho *et al.* (1) to have the effect of accelerating fermentation in bread dough. The effect was brought about by a 70%-ethanol-soluble and dialyzable fraction of the extract.

On the other hand, the present authors observed that the nondialyzable, heat-labile fraction of mushroom extract exhibits an oxidant-like effect on the physical properties of unfermented dough (2).

This study was undertaken to confirm the oxidative effect of mushroom extract on dough and to determine whether the effect is due to an enzymatic reaction, specifically tyrosinase (EC 1.10.3.1).

MATERIALS AND METHODS

Materials

Mushrooms (*Agaricus bisporus*) were purchased from the Morimoto Fungi

[1]Presented at the 59th Annual Meeting, Montreal, Oct. 1974.

Plant, Kyoto. Commercially milled, untreated strong flour with 11.6% protein (N × 5.7) and 0.41% ash on a 13.5% moisture basis was used.

Reagents

Commercial tyrosinase from mushroom (grade III, 2750 units/mg) was a product of the Sigma Chemical Co., St. Louis, Mo. Finally, 4-methylcatechol, p-cresol, N-ethylmaleimide (NEMI), phenylthiourea (phenylthiocarbamate, PTC), and "Difco" purified casein were purchased from Nakarai Chemicals Ltd., Kyoto.

Preparation of Mushroom Extract and Acetone Powder

Fresh mushrooms were washed with tap water and then with ice-cold distilled water. They were separated into pilei and stipes and extracted separately. The sample was homogenized in 1.2 times (volume to weight) of M/25 phosphate buffer, pH 7.0, and centrifuged at 10,000 × *g* for 60 min at 5° C. To the supernatant, subsequently referred to as "fresh extract," 1.5 volumes of cold acetone (−15° C) was added. The mixture was allowed to stand for 10 min in the cold and then centrifuged at 10,000 × *g* for 30 min. The precipitate was homogenized in the same volume of acetone as before, centrifuged, and lyophilized. A portion of the fresh pileus extract was dialyzed against water. The dialysate was concentrated under reduced pressure and stored at −20° C until use. Acetone powder was homogenized with M/25 phosphate buffer, pH 7.0, and centrifuged at 10,000 × *g* for 20 min before use. The supernatant is referred to as "acetone powder extract."

Extensigraph

Doughs were prepared with 300 g of flour, 180.3 ml of distilled water, 6.0 g of sodium chloride, and various additives in a Farinograph mixer. Doughs were mixed for 1 min, rested for 5 min, and remixed for 2 min. Potassium iodate was added in amounts of 1.5, 3.0, 4.5, and 6.0 mg per 300 g flour. The volume of fresh mushroom extract corresponding to 1.0 g dry weight of mushroom was calculated to be 13.7 ml for pileus extract and 10.3 ml for stipe extract. Equivalent amounts from 0.5 g to 3.0 g dry weight of mushroom were added to 300 g flour. Pileus acetone powder (254 mg), stipe acetone powder (316 mg), and commercial tyrosinase (7.4 mg) were calculated to be equivalent to 1.0 g dry weight of fresh mushroom stipe on the basis of tyrosinase activity toward 4-methylcatechol.

In some experiments, 37.5 mg (300 μmol) of NEMI or 8.3 mg (54 μmol) of PTC was added to 300 g flour. In the experiment using NEMI, dough was made by mixing 300 g flour with NEMI and 170.3 ml of water initially, resting for 5 min, and then remixing with 10 ml of acetone powder extract for 2 min. Two pieces of dough (150 g) were tested by an extensigraph according to the Brabender manual. Extensigrams were evaluated by the ratio of maximum resistance to extensibility at maximum resistance (R_{max}/E_{max}).

Estimation of Enzymatic Activities

Tyrosinase was determined electrometrically with the reaction medium containing 1.6 or 3.2 μmol of 4-methylcatechol (3) as substrate per 2.22 ml of total volume in 0.2*M* phosphate buffer, pH 7.0, at 30° C. In some experiments, p-

cresol (3) was used as substrate, and PTC ranging in concentration from $10^{-6}M$ to $10^{-4}M$ or $1.67 \times 10^{-3}M$ NEMI was added. Decrease in the oxygen concentration of the reaction mixture was recorded by using a galvanic cell, "Bioxygraph" (Kyusui Kagaku Lab. Co., Tokyo) and a recorder, Hitachi type 056. The activity was expressed as μmol oxygen consumed during 1 min. Proteolytic activity was determined according to Kunitz (4) by using casein as substrate.

RESULTS

Effect of Mushroom Extract and Iodate on the Extensigrams

Extensigrams with potassium iodate and with fresh extract of mushroom pileus are presented in Fig. 1. Addition of mushroom extract caused an increase in resistance and a decrease in extensibility. The effect is similar to that of an oxidizing agent. Comparison of Figs. 1a and 1b indicates that 0.5 g and 1.5 g of mushroom dry matter are approximately equivalent to 2 mg and 6 mg of iodate, respectively, on the extensigrams.

The effect of adding mushroom pileus, nondialyzable fraction of the extract, dialysate, or the extract reconstituted by mixing dialysate and nondialyzable fraction on extensigrams is shown in Fig. 2. Results show that the active component of mushroom extract causing a change in extensigrams is in the non-dialyzable fraction.

Effect of Mushroom Acetone Powder on Extensigrams

Figure 3 shows the extensigram data for doughs to which was added fresh extract of acetone powder equivalent to 2.0 g mushroom (dry weight) with respect to tyrosinase activity toward 4-methylcatechol.

Addition of both pileus and stipe extracts increases the resistance of dough to extension. However, the effect of stipe extract and its acetone powder is significantly more pronounced than that of corresponding preparations from the pileus.

Table I summarizes the values of R_{max}/E_{max} and tyrosinase activity of the samples.

There was a pronounced effect on extensigrams at the 45 min and the 90 min rest periods. Addition of stipe extract caused an increase in the resistance to extension when the rest period was increased from 45 min to 90 min. On the other hand, addition of pileus resulted in similar extensigrams when the 45 min rest period was changed to 90 min.

The fact that stipe and pileus samples, equivalent on the basis of tyrosinase activity toward 4-methylcatechol, act in a different manner on an extensigraph suggests that constituents of the extract other than or in addition to tyrosinase affect the physical properties of dough.

Effect of Mushroom Acetone Powder in the Presence of Phenylthiourea

Tyrosinase activity of mushroom acetone powder toward p-cresol and 4-methylcatechol was completely inhibited with $10^{-4}M$ PTC, as seen in Table II.

Acetone powder extract previously treated with $3 \times 10^{-4}M$ PTC for 10 min at 30°C was added to dough. The extensigrams are presented in Fig. 4.

No further effect of stipe powder was detected when it was pretreated with

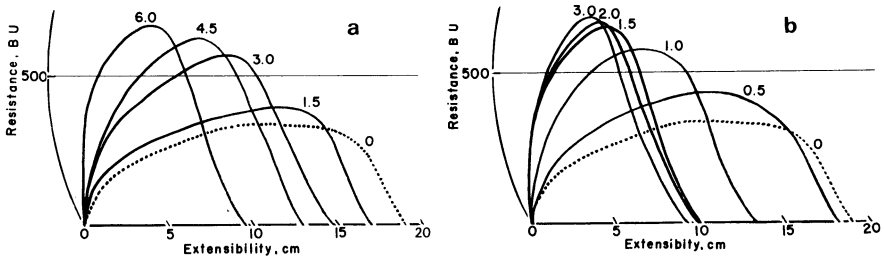


Fig. 1. Effect of potassium iodate or mushroom extract on extensigram. a) Potassium iodate in amounts ranging from 1.5 to 6.0 mg was added to 300 g flour. b) Mushroom extracts which were obtained from the fresh samples equivalent to the amount of dry weight ranging from 0.5 to 3.0 g.

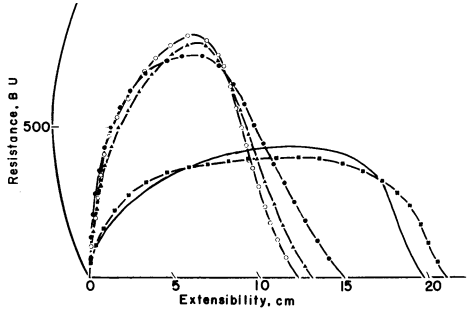


Fig. 2. Effect of mushroom extract with or without dialysis on the extensigram. Each mushroom sample corresponding to 2.0 g of dry matter of fresh pileus was used for 300 g flour. Dough control ——; dough with crude extract •——•; dough with dialyzed extract ▲——▲; dough with dialyzed extract plus dialysate o——o; dough with dialysate ■——■.

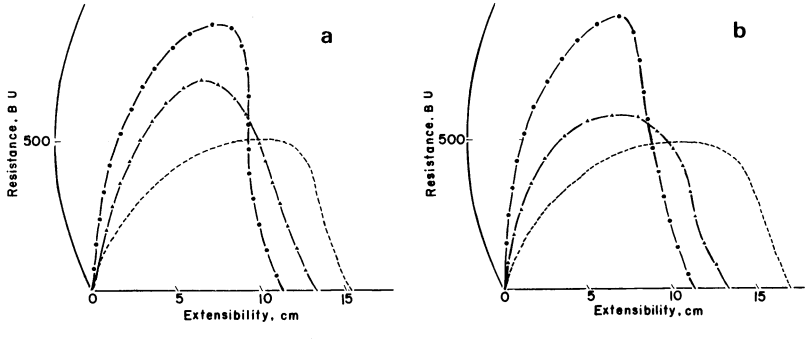


Fig. 3. Effect of crude extract and acetone powder extract of mushroom on extensigram. a) Crude extract (27.4 ml for pileus or 20.6 ml for stipe) was used. b) Acetone powder (507 mg for pileus or 631 mg for stipe) was used. Dough control - - - - -; dough with pileus extract ▲——▲; dough with stipe extract •——•.

PTC, though PTC alone has little effect increasing resistance to extension as compared with the control experiment. In the case of pileus it had a negative effect in decreasing the ratio of R_{max} to E_{max}.

Effect of Mushroom Acetone Powder in the Presence of N-Ethylmaleimide

Extensigrams with NEMI and mushroom extracts are shown in Fig. 5.

Pretreatment of flour with NEMI masks the effect caused by mushroom extract. The addition of stipe extract made no difference in the extensigrams of NEMI-pretreated dough. However, the addition of pileus extract to NEMI-pretreated dough resulted in a much smaller ratio as compared with the extensigram of the NEMI-pretreated dough control. Tyrosinase activity of the mushroom samples was not affected by $1.67 \times 10^{-3} M$ NEMI, as shown in Table III.

TABLE I
Extensigraph Parameters of Doughs with Mushroom Extract and Total Activity of Tyrosinase in the Extract

Experiment	Extensigraph Parameters		Tyrosinase Activity toward 4-Methylcatechol[a]
	45 min	90 min	
	BU/cm	BU/cm	μmol O_2/min
Control	34.7	41.6	...
Crude extract			
Pileus	65.2	83.0	314
Stipe	66.4	106.6	429
Acetone powder			
Pileus	49.3	67.4	435
Stipe	83.3	135.7	436

[a]The activity was expressed as that contained in the volume which was used for 300 g flour. Tyrosinase activity was measured in the medium containing 1.6 μmol of 4-methylcatechol and mushroom extract in 2.22 ml of total volume.

TABLE II
Effect of Phenylthiourea on the Tyrosinase Activity of Mushroom Stipe Extract

Experiment	Tyrosinase Activity[a]			
	Toward p-cresol		Toward 4-methylcatechol	
	μmol O_2 $min^{-1} \cdot mg^{-1}$	%	μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$	%
Extract				
Alone	0.156	(100.0)	0.549	(100.0)
With PTC, $10^{-6} M$	0.037	(23.7)	0.273	(49.7)
With PTC, $10^{-5} M$	0.009	(5.9)	0.057	(10.3)
With PTC, $10^{-4} M$	0	(0)	0	(0)

[a]Reaction was carried out in the medium containing 3.2 μmol of p-cresol or 4-methylcatechol and stipe acetone powder extract with or without PTC in 2.22 ml of total volume.

Extensigraph of Dough with Purified Tyrosinase

Results with purified tyrosinase, a commercial product used in an amount equivalent to that of the acetone powder on the basis of its activity to catechol, are summarized in Fig. 6. NEMI was used in a manner similar to that in the previous experiment for acetone powder.

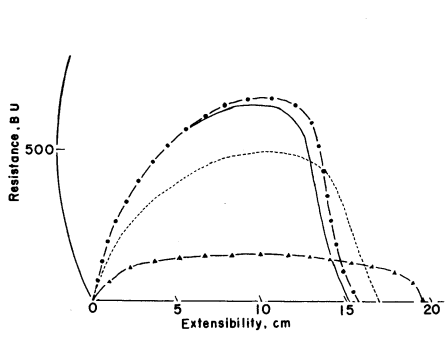
The purified enzyme exhibited an oxidative effect on the extensigram and, although not shown in the figure, increased the R_{max}/E_{max} ratio with increasing rest period.

On doughs treated with NEMI, the enzyme caused no further effect than that with NEMI alone. The behavior of purified tyrosinase on the extensigram is comparable with that of mushroom stipe, as seen in Figs. 4 and 5.

TABLE III
Tyrosinase Activity of Mushroom Acetone Powder and Purified Tyrosinase in the Presence of N-Ethylmaleimide

Experiment	Tyrosinase Activity toward 4-Methylcatechol[a]	
	Without NEMI	With NEMI
	μmol O_2 $min^{-1} \cdot mg^{-1}$	
Acetone Powder		
Pileus	1.04	1.24
Stipe	1.45	1.45
Purified Tyrosinase	31.4	30.6

[a]Tyrosinase activity was measured in the medium containing 3.2 μmol of 4-methylcatechol, 2.31 mg of NEMI ($1.67 \times 10^{-3} M$), and sample in 2.22 ml of total volume.



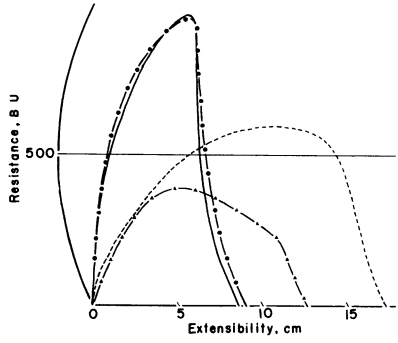


Fig. 4. Effect of mushroom acetone powder extract in the presence of phenylthiourea on extensigram. Extract from 507 mg of pileus powder or 631 mg of stipe powder and PTC (8.3 mg, 54 μmol) was preincubated in 180.3 ml of water. Dough control - - - - -; dough with PTC _____; dough with PTC plus pileus ▲_____▲; dough with PTC plus stipe •_____•. Fig. 5. Effect of N-ethylmaleimide and mushroom acetone powder extract on extensigram. To dough pretreated with 37.5 mg of NEMI, mushroom acetone powder extract was added (507 mg for pileus or 631 mg for stipe). Dough control - - - - -; NEMI-dough _____; NEMI-dough plus pileus extract ▲_____▲; NEMI-dough plus stipe extract •_____•.

Tyrosinase Activity toward P-Cresol and Proteolytic Activity of Mushroom Extract

Proteolytic enzyme and tyrosinase activities of acetone powder extracts are presented in Table IV. Proteolytic activity is higher in the pileus than in the stipe at both pH values, whereas tyrosinase activity toward p-cresol of pileus is slightly higher than that of stipe.

DISCUSSION

The fact that the nondialyzable fraction of mushroom extract had an oxidative effect on the extensigraph of dough as shown in Fig. 2, an effect that was lost by the heating of extract at 98°C for 3 min and was rapidly diminished in the mushroom tissue during storage even under low temperature (although experimental data are not shown here), leads the authors to suggest that the effective component of mushroom is an enzyme.

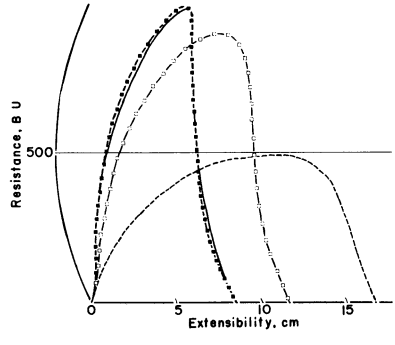


Fig. 6. Effect of N-ethylmaleimide and purified tyrosinase. To dough pretreated with 37.5 mg of NEMI, 14.8 mg of tyrosinase was added. Dough control - - - - -; NEMI-dough ——; Tyrosinase-dough □——□; NEMI-dough plus tyrosinase ■——■.

TABLE IV
Enzyme Activity in Acetone Powder used for Extensigraph[a]

Experiment	Activity	
	Pileus	Stipe
Tyrosinase		
for p-Cresol	170	158
for 4-Methylcatechol	436	435
(μmol $O_2 \cdot min^{-1}$)		
Proteolytic enzyme		
pH 5.5	6.08	5.05
pH 7.0	1.01	0.63
($E_{275\ nm}$/min)		

[a]507 mg for pileus and 631 mg for stipe were used for 300 g flour.

The difficulty that the magnitude of the effect varied from sample to sample was overcome by employing an acetone powder of the extract. The powder could be stored in a desiccator at −20° C for over 100 days with no loss of effect on dough and no loss of enzymatic activity.

In the experiment summarized in Fig. 3 and Table I, amounts of acetone powder extract of pileus and stipe were employed such that they were at a similar level in regard to tyrosinase activity. Nevertheless, the magnitude of the effect on dough was significantly different between pileus and stipe.

This difference in extensigram response is confirmed further in Figs. 4 and 5. Treatment of the pileus extract with PTC, a tyrosinase inhibitor, and pretreatment of flour with NEMI caused an effect on extensigram much like that of a reducing agent or protease. It would appear that there are two factors in whole mushrooms that alter dough properties. Tyrosinase exhibits an oxidative effect associated with SH-groups in flour, while another component in the pileus, insensitive to tyrosinase inhibitor, appears to dominate dough characteristics independent of SH-groups in flour.

In general, proteases weaken dough, decreasing the ratio of R_{max}/E_{max} on extensigrams. Thus it might be suggested that the effect on the dough property of the pileus extract is partly due to proteases. This, however, must be investigated further.

In addition, the different effect on extensigrams of pileus and stipe extract shown in Fig. 3 might be partly due to the difference in cresolase activity between both extracts, since they were added to dough so that their amounts would have the same levels of tyrosinase activity to catechol (catecholase activity). However, the small difference in tyrosinase activity to cresol is seen between pileus and stipe.

The hypothesis that the endogenous tyrosinase of mushroom acts as a powerful dough-improver was proved by the experiment in which purified tyrosinase was used instead of mushroom extract (Fig. 6).

Tyrosinase has been known to have a wide substrate specificity and to exist as a multiple form, oxidizing a monophenol to a corresponding o-diphenol and a diphenol to a corresponding o-quinone as shown in the next scheme.

Scheme: Two-step reaction of tyrosinase

$$\text{Monophenol} \xrightarrow[\text{Cresolase Activity}]{+\,1/2\,O_2} \text{o-Diphenol} \xrightarrow[\text{Catecholase Activity}]{+\,1/2\,O_2} \text{o-Quinone}$$

The capacity of aqueous extract of flour to form viscoelastic gel in the presence of an oxidizing agent was observed long ago (5), and later it was proposed by Fausch *et al.* (6) that oxidation of ferulic acid ester groups attached to a pentosan chain is implicated in the gelation mechanism of pentosans. The dough-strengthening effect of tyrosinase shown above may relate to the oxidation of the pentosan fraction of flour through the ferulic acid moiety to the corresponding quinone; however, this has not yet been clarified.

Tyrosinase has been widely used for studies on the conformation of proteins since it oxidizes reactive tyrosine residues in protein molecules (7) and, further, it

has been shown that the oxidation product of phenol by tyrosinase forms an adduct with thiol (8). Tyrosinase may bring about a change in the macroscopic structure of a gluten matrix through oxidation of tyrosine residues in the gluten proteins. Also, the resulting conjugation between oxidized products of tyrosine and flour thiols may change the physical properties of dough, since tyrosine residues were measured at 20 mol/10^5 g gluten (9), and free tyrosine at 10 μmol/100 g flour (10).

CONCLUSION

It has been confirmed that the oxidative effect of mushroom extract on dough is due to endogenous tyrosinase. Further study is necessary to elucidate the specific role of tyrosinase in mushroom extract.

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