Phytochemistry, 1977, Vol. 16, pp. 1351–1354. Pergamon Press. Printed in England.

ANION ACTIVATION OF γ-GLUTAMYLTRANSFERASE FROM FRUITING BODIES OF *LENTINUS EDODES*

KYODEN YASUMOTO, KIMIKAZU IWAMI, TOSHIHIKO YONEZAWA and HISATERU MITSUDA

Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606, Japan

(*Revised received* 17 *February* 1977)

Key Word Index—*Lentinus edodes*; Basidiomyceteae; shiitake mushroom; γ-glutamyltransferase; halide activation; lentinic acid.

Abstract—Activation by different anions of γ-glutamyltransferase obtained in a particulate form from fruiting bodies of *Lentinus edodes* has been studied using either L-γ-glutamyl-*p*-nitroanilide or lentinic acid as substrate. The mushroom transferase was activated by SCN^-, NO_3^-, Cl^-, Br^-, ClO_3^-, BrO_3^-, N_3^-, I^- and F^-, but not those alkali and earth cations previously believed to activate the animal transferase, nor by citrate, claimed to be effective for the kidney bean transferase. Among anions proved hardly to activate the transferase were ClO_4^-, NO_2^-, HCO_3^-, $H_2PO_4^-$, SO_3^{2-} and SO_4^{2-}. A high concentration of these anions more or less impeded the halide activation. Kinetic studies revealed that halides function as activators of increasing V_{max} while keeping K_m constant. These observations appeared least compatible with the possibility that the anion activation might involve a non-specific effect of high solute concentration, *viz.* dissociation of the enzyme from the supporting structure in the particulates. The activating effect of halides described here probably extends also to the animal enzymes.

INTRODUCTION

Previous work from this laboratory [1] has established that a unique γ-glutamyl peptide occurring in an edible Basidiomyceteous mushroom *Lentinus edodes* is the precursor of lenthionine (1,2,3,5,6-pentathiepane), which has been identified as a characteristic aroma-bearing substance of the mushroom [2, 3]. It has also been demonstrated that γ-glutamyltransferase (EC 2.3.2.2) catalyzes the initial step in the aroma evolution from this peptide called trivially lentinic acid [4, 5].

In animal tissues γ-glutamyltransferase is closely associated with membrane fractions and localized at sites where high uptake of amino acids occurs [6, 7]. Alkali metal cations, together with Mg^{2+}, are activators for the kidney transferase [8]. These observations have permitted speculation on the physiological function of the transferase [6–8]. In a previous report [4], we postulated that the mushroom transferase activity is dependent on the type and the concentration of the buffer employed in the reaction medium; the activity increases or decreases respectively with increasing concentration of Tris–HCl or phosphate buffer. We have reappraised these findings and have found that activation involves monovalent anions most and cations least, and is probably ascribable to a specific interaction of the anions with the enzyme protein.

RESULTS

γ-Glutamyltransferase from *L. edodes* exhibited only a marginal activity in 0.1 M Tris–acetate–sodium citrate buffer over the pH range of 7.3 to 10. Addition of NaCl to the reaction mixture produced a dramatic activation of the transferase to give such tangible and normal shaped pH-activity profiles as shown in Fig. 1. The pH-activity profiles appear to be of identical form and position, differing only in height by the concentration of NaCl added as activator. Removal of sodium citrate from the

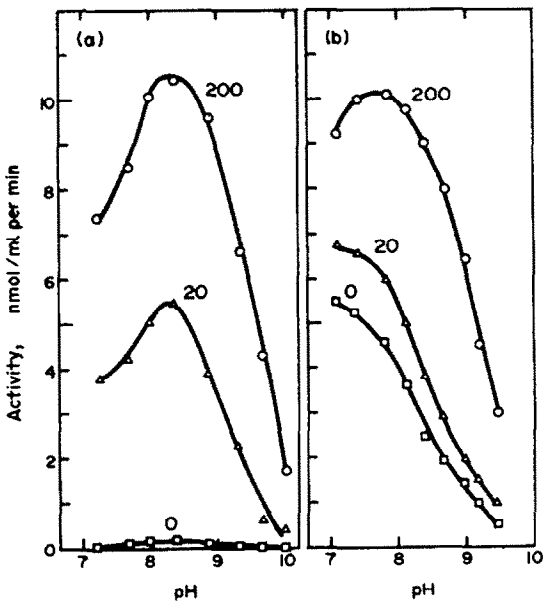

Fig. 1. pH-activity profiles of *Lentinus* γ-glutamyltransferase in the presence and absence of NaCl. The enzyme activity was assayed in either 0.1 M Tris–acetate–sodium citrate or 0.1 M Tris–HCl buffer at the indicated pH values. Concentrations of the substrate and the enzyme protein in the final assay mixture are the same as described for Table 1. The millimolar concentration of added NaCl is indicated over the curves.

Table 1. Effect of different cations on *Lentinus* γ-glutamyltransferase activity

Salts	Activity (nmol/min)		
	10 mM	30 mM	100 mM
LiCl	6.6	11.2	15.5
NaCl	7.2	11.4	16
KCl	7.7	12	16.4
RbCl	7.6	12.1	16.3
$CaCl_2$	9.9	14.5	18.3
$MgCl_2$	10	14.3	18

The assay mixture (final vol. 1 ml) contained the indicated concentration of chloride salts and 10 μg of enzyme protein in the basal assay mixture. The enzyme activity was determined with γ-glutamyl-*p*-nitroanilide and defined as described under Experimental.

assay mixture had little effect on the transferase activity. It thus follows that neither Na^+, being previously claimed as a potent activating cation for the animal transferase [8], nor citrate, known to formulate a most efficient activator pair with Na^+ for the kidney bean transferase [9], activates the mushroom transferase. Hence the activation by added NaCl proved ascribable to a specific effect of the anion Cl^-.

With Tris–HCl buffer the activity of the mushroom transferase is a function of the buffer concentration [4] approaching a constant level at higher concentrations. This hyperbolic relationship is one of the characteristics molecular interactions describable in terms of the law of mass action. Fig. 1 shows the effect of varying the pH of Tris–HCl buffer in the assay medium on the transferase activity in the presence or absence of NaCl. Incorporation of 0.2 M NaCl into the reaction mixture produces a shift of the pH optimum toward the alkaline side (from pH 7.1 to 7.7). This results from the greater activation by NaCl at higher pH as a consequence of the smaller concentration of HCl used as acid against the constant Tris concentration, and hence of lesser transferase activity in the absence of added NaCl with the buffer of higher pH.

Table 1 compares the effects of some alkali metal and alkali earth chlorides on the mushroom transferase when incorporated with the basal assay mixture. Alkali earths appeared to be slightly more effective than alkali metals; this merely reflects however bivalencies of alkali earths. It thus follows that the transferase activity is not dependent on the nature of cations in the reaction medium.

The specificity of the above-inferred anion effect as activator of the mushroom transferase was examined using sodium salts of several inorganic anions in the basal assay mixture (Table 2). Among the most effective activators with the synthetic substrate were Cl^- and Br^- besides SCN^- and NO_3^-, while I^- and F^- were weak activators; ClO_3^-, BrO_3^- and N_3^- activated the transferase to some extent, but ClO_4^- to the least extent. A similar anion activation was also observed with the natural substrate lentinic acid. The degree of activation, varying with the anion used, was in general much higher than that with synthetic substrate. It thus appears that the effectiveness varies with the substrate used in the assay. Acetate and citrate, at the concentration contained in the basal assay mixture, had little effect on the enzyme activity. Besides these organic anions, NO_2^-, HCO_3^- (plus CO_3^{2-}), SO_3^{2-}, SO_4^{2-} and $H_2PO_4^-$ (plus HPO_4^{2-}) failed to substitute for halide anions. It is noteworthy that sodium thiocyanate, well-known as a typical chaotropic agent, activated most strongly the mushroom transferase but its activity was exclusively found in the 105000 *g* pellet after preincubation with the chaotropic anion.

Conventional double reciprocal plots of the velocity data obtained with the basal assay mixture containing different fixed concentrations of NaCl gave a family of straight lines crossing at a point on the 1/S axis, indicating that Cl^- does not affect the apparent K_m value of

Table 2. Anion activation of *Lentinus* γ-glutamyltransferase with synthetic and natural substrates

Salts	Activity with substrate (nmol/min)	
	γ-Glutamyl-*p*-nitroanilide	Lentinic acid
No addition	0.2	7.7
NaF	4.5	71
NaCl	(16)	113.4
NaBr	(15.5)	107.3
NaI	4.5	90.3
NaN_3	5.8	91.3
$NaNO_2$	0.6	
$NaNO_3$	(22)	92.7
NaSCN	(27.4)	76.8
$NaClO_3$	8.6	65.5
$NaBrO_3$	8.4	
$NaClO_4$	0.4	55.2
Na_2SO_4	0.3	
Na_2HPO_4	0.5	

Assay conditions are the same as in Table 1 except that the assay mixture contained the shown sodium salts at 100 mM in place of the chloride salts and either one of the two substrates. Data in parentheses are from Fig. 2.

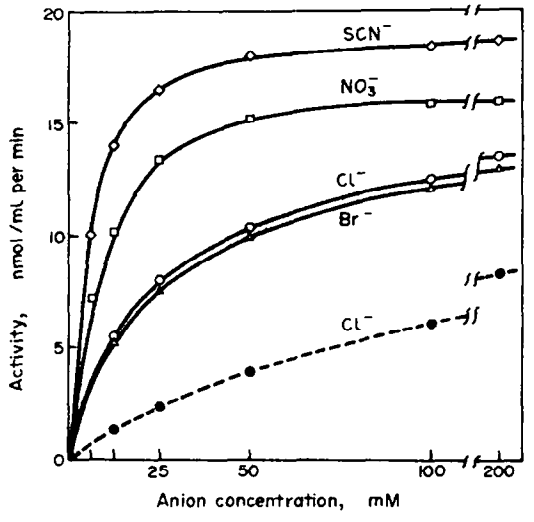

Fig. 2. Effect of typical activating anions at varied concentrations on *Lentinus* γ-glutamyltransferase activity. The enzyme activity was assayed in either 0.1 M Tris–acetate–sodium citrate (solid lines) or 0.1 M Tris–H_3PO_4 buffer (broken line) of pH 8.3. Other assay conditions are the same as given in Table 1, except for varied concentrations of anions.

about 5 mM but increases the V_{max} value of the transferase reaction (data not shown). In Fig. 2 are shown the effects on the mushroom transferase activity of typical anions at varied concentrations added to the basal assay mixture (solid lines). The curve of activation by SCN^- and NO_3^- reached a plateau at relatively low concentrations. At the higher concentrations of Cl^- (or Br^-) the transferase was much more activated. The activating effect of halides beyond 0.2 M was however inferior to that of SCN^-. When the reciprocals of velocity data were plotted against those of the corresponding anion concentrations, the intercept on the horizontal axis should be rather regarded, although not involving any function of K_m, as representing the reciprocal of the affinity constant of the anion for the transferase. The values thus obtained, affinity constants, were *ca* 6, 10 and 20 mM for NaSCN, $NaNO_3$ and NaCl (or NaBr), respectively.

Phosphate is a weak inhibitor [4] and thus would be expected to compete with other activator anions when added together. As further shown in Fig. 2 (broken line), addition of NaCl to 0.1 M Tris–H_3PO_4 buffer resulted in activation, but to a lesser extent than that observed with the basal assay mixture, while giving the intercept of the same V_{max} value as with the basal assay mixture containing NaCl. This apparent competition effect suggests that phosphate competes with Cl^- for a common site on the enzyme.

DISCUSSION

The effect of halide anions on the kinetics of γ-glutamyltransferase from fruiting bodies of *L. edodes* is to increase impressively the catalytic activity without affecting the affinity of the enzyme towards the substrate. When Cl^- was activator, apparent K_m for the substrate γ-glutamyl-*p*-nitroanilide remains virtually unchanged while V_{max} increases as a function of added Cl^- concentration. Conversely it follows that the substrate should not affect the binding of the anions to the enzyme as is also verified experimentally in this study.

γ-Glutamyltransferase of animal origin has been found to depend on various inorganic ions. Orlowski *et al.* [8] reported that the transferase from kidneys and choroid plexuses of several animals were strongly activated by alkali metal ions. Nevertheless, as their work was fortuitously conducted in Tris–HCl buffer and with the use of chloride salts as test compounds, their interpretation of the experimental results is hardly tenable but should be reevaluated in terms of Cl^- activation.

Elce *et al.* [10] also observed a marked activating effect of added chloride salts but interpreted the results in terms of activation by Cl^- together with alkali metal cations, sticking to the conventional idea of those days [8]. Miller *et al.* [11] have recently suggested that typical members of alkali metal and alkali earth cations had no appreciable effect on the activity of the human kidney transferase. These may support the concept that the specificity of the activation of animal transferase involves the anions, as is demonstrated for the mushroom enzyme, but not the cations on any account. In a previous report [4], data were presented to suggest that the activation of the mushroom transferase is dependent on the ionic strength of the reaction medium. These data were obtained in the presence of Cl^- fortuitously added as buffer component, hence should be accounted for by a specific activating effect of Cl^-. The kinetic behaviour of the mushroom transferase described in this paper is compatible with the existing concepts which ultimately postulate that the binding of the activator to its specific site causes a conformational change in the enzyme molecule. Additionally, several lines of circumstantial evidence afford a reasonable basis for the central idea of the argument in issue that anion activation of the mushroom enzyme is mostly attributable to conformational alteration of the enzyme protein and little if not least to the structural change or dissolution of the membrane to which the enzyme is bound. Activation by anions of the animal transferase is not simply confined to the bound enzyme but extends to the soluble enzyme [10]. This may also apply to the mushroom transferase. Detergents did not produce appreciable activation or solubilization of the enzyme [4]. Moreover, ClO_4^-, second rank anion in Hoffmeister series, showed the least activating effects on the mushroom enzyme.

In all of these and other respects, the anion effect proves to involve a specific interaction with the enzyme protein, but not accompanied by association nor by dissociation of the enzyme subunit from a supporting structure to which the enzyme (or its possible subunits) is bound.

Recent studies on the mechanism of anion activation of α-amylase [12], dopamine-β-hydroxylase [13] and adenyl cyclase [14] should be especially considered in relation to the anion activation of the transferase. The binding of anions to a specific binding site adjacent to the ionizable group at the active site, has been proposed to explain the anion activation, a single ε-amino group of lysine being identified as the Cl^- binding site of α-amylase [12]. The present results warrant inclusion of the mushroom transferase in the class of those enzymes showing activation by anions, and allow them to be considered as models for the mushroom and possibly also for the animal transferase. However, the present data are not yet conclusive as to the molecular mechanism whereby the anion activation is effected. Its elaboration must await the isolation of the soluble transferase.

EXPERIMENTAL

γ-Glutamyltransferase was obtained in microsomal fractions from caps of the mushroom, *L. edodes*, successively by differential centrifugation at 5000 *g* for 15 min, at 20000 *g* for 15 min 105000 *g* for 1 hr [4]. The enzyme activity was determined by measuring the release at 37° for 10 min of *p*-nitroaniline from the synthetic substrate L-γ-glutamyl-*p*-nitroanilide, or of pyruvate from the natural substrate lentinic acid in the presence of an excess of C-S lyase [5]. Most of the assays were performed with 2 mM substrate and *ca* 10 μg of the enzyme protein in 1 ml of 0.1 M Tris–acetate–Na citrate buffer, an equimolar mixture of Tris and Na citrate adjusted to pH 8.3 with HOAc (basal assay mixture). Actual assay conditions under which pH dependence, ion specificity, kinetics, etc. were studied are given in the text. The enzyme activity was expressed in terms of nmol of *p*-nitroaniline or pyruvate released per min.

REFERENCES

1. Yasumoto, K., Iwami, K. and Mitsuda, H. (1971) *Agr. Biol. Chem.* 35, 2059, 2070.
2. Morita, K. and Kobayashi, S. (1966) *Tetrahedron Letters* 773.
3. Morita, K. and Kobayashi, S. (1967) *Chem. Pharm. Bull.* 15, 988.
4. Yasumoto, K., Iwami, K. and Mitsuda, H. (1974) *Proceedings of the IXth International Scientific Congress on the Cultivation of Edible Fungi*, the Mushroom Research Institute, Kiryu/Tokyo, Japan. p. 371.

5. Iwami, K., Yasumoto, K., Nakamura, K. and Mitsuda, H. (1975) *Agr. Biol. Chem.* **39**, 1933, 1941.
6. Meister, A., Tate, S. S. and Ross, L. L. (1976) *The Enzymes of Biological Membranes*, Vol. 3 (Martonori, A., ed.) pp. 315. Plenum Press, New York.
7. Orlowski, M. and Meister, A. (1970) *Proc. Natl. Acad. Sci. U.S.* **67**, 1248; Meister, A. (1973) *Science* **180**, 33.
8. Orlowski, M., Okonkwo, P. O. and Green, J. P. (1973) *FEBS Letters* **31**, 237.
9. Goore, M. Y. and Thompson, J. F. (1967) *Biochim. Biophys. Acta* **132**, 27.
10. Elce, J. S., Bryson, J. and McGirr, L. G. (1974) *Can. J. Biochem.* **52**, 33.
11. Miller, S. P., Awasthi, Y. C. and Srivastava, S. K. (1976) *J. Biol. Chem.* **251**, 2271.
12. Levitzki, A. and Steer, M. L. (1974) *European J. Biochem.* **41**, 171.
13. Craine, J. E., Daniels, G. H. and Kaufman, S. (1973) *J. Biol. Chem.* **248**, 7838.
14. Kalish, M. I., Pineyro, M. A., Cooper, B. and Gregerman, R. I. (1974) *Biochem. Biophys. Res. Commun.* **61**, 731.