Enzymatic Conversion of γ-L-Glutamyl Cysteine Peptides to Pyruvic Acid, A Coupled Reaction for Enhancement of Onion Flavor

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Part of the flavor precursors in onion are present as γ-L-glutamyl peptides and are thus insusceptible to the action of the endogenous flavor-producing enzyme. Both *Albizzia* L-cysteine C–S lyase and kidney transpeptidase acting sequentially in a coupled reaction are required for the production of pyruvic acid (a concomitant of enzymatic flavor development) from γ-L-glutamyl-*S*-methyl-L-cysteine. Under optimal conditions for the action of C–S lyase on the cysteine derivatives present in the cation fraction of onions, no pyruvic acid was produced by transpeptidase in the absence of C–S lyase. In the presence of the latter, transpeptidase enhanced pyruvic acid production beyond that formed in the presence of the lyase alone. These results suggest that fortification of onion-containing foods with appropriate enzyme adjuncts may elicit the full flavor potential of such foods.

The γ-L-glutamyl transpeptidases [E.C. 2.3.2.1] catalyze the transfer of the glutamyl moiety of a γ-L-glutamyl peptide to a second amino acid.

γ-L-glutamyl amino acid$_1$ + amino acid$_2$ ⟶

γ-L-glutamyl amino acid$_2$ + amino acid$_1$ (1)

This enzyme is widely distributed and is especially abundant in mammalian kidney (Orlowski and Meister, 1970). It is absent in unsprouted onions (Austin and Schwimmer, 1971) which contain a variety of γ-L-glutamyl peptides. A quantitatively large fraction of these peptides contains amino acids which are precursors to onion flavor, the *S*-propenyl and *S*-methyl derivatives of L-cysteine sulfoxide, as well as *S*-methyl-L-cysteine (Matikkala and Virtanen, 1967).

Since kidney transpeptidase also catalyzes the hydrolysis as well as glutamyl transfer, the addition of this enzyme to onion should liberate the flavor precursor.

γ-L-glutamyl-*S*-alk(en)yl-L-cysteine (sulfoxide) + H_2O ⟶

L-glutamic acid + *S*-alk(en)yl-L-cysteine (sulfoxide) (2)

The free cysteine sulfoxide derivatives should thus be susceptible to conversion to the volatile sulfur flavor components of onions by either the endogenous L-cysteine sulfoxide lyase (E.C. 4.4.1.4.; Schwimmer, 1970, 1971) or by a suitable exogenous source of enzyme such as the L-cysteine C–S lyase of *Albizzia lophanta* seeds (Schwimmer and Kjaer, 1960; Schwimmer and Guadagni, 1968). This enzyme can act on S-substituted derivatives of both cysteine and cysteine sulfoxide.

S-alk(en)yl-L-cysteine (sulfoxide) + H_2O ⟶

$S_{(volatile)}$ + NH_3 + pyruvic acid (3)

Coupling of eq 2 to eq 3 should lead to the concomitant liberation of both pyruvic acid and volatile sulfur flavors and thus enhance onion flavor.

In the present communication we show that this coupled reaction

γ-L-glutamyl-*S*-alk(en)yl-L-cysteine (sulfoxide) + $2H_2O$ ⟶

L-glutamic acid + $S_{(volatile)}$ + NH_3 + pyruvic acid (4)

can be demonstrated with suitable enzyme preparations, with both synthetic peptides and with the cationic eluate fraction from onion as substrates. These studies have been designed as the basis for further developmental investigation leading to appropriate commercial application.

MATERIALS AND METHODS

Substrates. Substrates for transpeptidase, γ-L-glutamyl peptides of nitroaniline (GNA), and of *S*-methyl-L-cysteine (GMC) were synthesized as previously described (Austin and Schwimmer, 1971). The substrate for assessing the activity of C–S lyase was synthetic (±)-*S*-propyl-L-cysteine sulfoxide (Schwimmer, 1971). Cation exchange eluates employed as substrates for these enzymes were prepared by combination of the methods of Carson *et al.* (1966) for the preparation of the boiled filtered extract of dehydrated onion powder and that of step 2 of Schwimmer (1969) for the isolation of *S*-propenyl-L-cysteine sulfoxide. Fractions possessing the ability to serve as substrates for C–S lyases were combined and the pH adjusted to 7.5. The combined eluates contain the γ-L-glutamyl peptides as well as the amino acids of onion (Carson *et al.*, 1966).

Two different onion powders were subjected to these procedures. Their neutralized cation exchange eluates are designated as A and B. One milliliter of A or B corresponds to 0.22 or 0.39 g of onion powder, respectively. Substrate preparations A′ and B′ are water suspensions of the onion powders clarified by centrifugation and filtration.

Enzyme Preparation and Assay. Transpeptidase was prepared from 1 g of kidney acetone powder (Pentex). After dispersion of the powder in 20 parts of cold water, the resulting suspension was purified through the desoxycholate step of Orlowski and Meister (1965) and then dialyzed and freeze-dried to yield 90 mg of enzyme preparation, designated as T (Table I). Preparation T had an activity of 572 transpeptidase units $(U)_t$ per mg. One unit of transpeptidase activity is defined as that amount of enzyme that will liberate 1 μmol of *p*-nitroaniline per minute from γ-L-glutamyl nitroanilide under standard conditions previously described (Austin and Schwimmer, 1971). The C–S lyase activity (see below) was less than 0.3 U_1 per mg.

L-Cysteine C–S lyase was prepared from the seeds of the flowering shrub *Albizzia lophanta* according to the procedure of Schwimmer and Guadagni (1968). The specific activity

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of this enzyme preparation (L) was 54 units (U_l) per mg. One unit of lyase activity will produce 1 μmol of pyruvic acid from (±)-*S*-propyl-L-cysteine sulfoxide in 10 min under previously defined standard conditions (Schwimmer, 1971). Pyruvic acid was determined according to a previously published procedure (Schwimmer and Weston, 1961).

Criterion for the Coupling Reaction. The combined action of enzyme preparations T and L was detected by the increase in pyruvic acid production upon their simultaneous or sequential addition to enzyme reaction mixtures containing peptide. All experiments were conducted at 37° C in the presence of toluene for incubations longer than 1 hr. Unless otherwise stated, each milliliter of reaction mixture contained 0.04 *M* pyrophosphate buffer pH 8.2 and 0.05 m*M* pyridoxal phosphate. Blanks containing enzyme alone and substrate alone were run and the values subtracted to yield "Δ" pyruvate. For the most part, results are expressed as Δ pyruvate per gram of onion powder. For convenience and ease of reference, the variable conditions used in the ensuing experiments are shown in Table I.

RESULTS AND DISCUSSION

Effect of Optimum Lyase Conditions on Transpeptidase. Previous work has shown that C–S lyases act optimally at pH 8 in the presence of pyrophosphate and pyridoxal phosphate (Schwimmer, 1971; Schwimmer and Kjaer, 1960), whereas kidney transpeptidase acts optimally in Tris buffer at pH 9 in the presence of Mg^{2+}. Table II shows that at pH 8 preparation T was appreciably activated by pyrophosphate and marginally but reproducibly activated by pyridoxal phosphate. It is of interest to note that Mg^{2+} activated the enzyme to about the same extent as did pyrophosphate at pH 8 to yield about the same activity as Tris + Mg^{2+} at pH 9. From these data we may conclude that the conditions for optimal lyase activity do not depress the action activity of transpeptidase preparation T acting on GNA.

Coupled Reaction with GMC as Substrate. In the absence of C–S lyase, transpeptidase did not catalyze the production of pyruvic acid from GMC (Figure 1). Conversely, no pyruvate was formed in the presence of lyase alone. With the two enzymes acting together simultaneously, pyruvic acid was produced at a constant rate of 2.5% per min until almost the entire 0.6 μmol/ml of the added GMC was converted. That these data constitute good evidence for a coupled reaction as depicted in eq 4 is corroborated by the effect of the order of addition (when one enzyme is omitted for 20 min) on the subsequent rate when the second enzyme is added. When lyase was added first, presumably nothing happened because the GMC is not a substrate for lyase action. Upon the addition of transpeptidase, the subsequent production of pyruvic acid paralleled that produced when the two enzymes were added simultaneously. When transpeptidase was added first, it presumably produced *S*-methyl-L-cysteine (see eq 2). The higher level of this substrate available to the lyase, added at 20 min, would result in an increased rate of pyruvic acid production. This prediction is borne out by the observed tripling of the rate of pyruvic acid production. We may conclude that concomitant addition of these two enzymes results in a coupled reaction sequence as represented in eq 2 to 4.

Onion Eluates as Substrates for C–S Lyase. Quantitative data have been reported which demonstrate that substrates for *Albizzia lophanta* C–S lyase are present in frozen (Schwimmer and Guadagni, 1968) and in fresh onion (Schwimmer, 1969). No such data have been reported for the action of this enzyme on dehydrated onion, although the latter has been used to isolate onion flavor precursors (Carson *et al.*, 1966). Furthermore cation eluates of onion have been shown to contain γ-L-glutamyl peptides. Data delineating the action of lyase preparation on such eluates are set forth

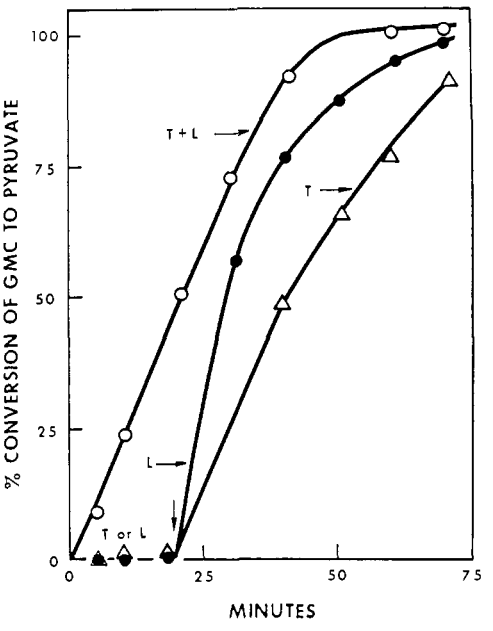

Figure 1. Coupling of C–S lyase (L) to transpeptidase (T) as measured by the production of pyruvic acid from γ-L-glutamyl-*S*-methyl-L-cysteine (GMC)

Table I. Experimental Variables

Experiment	Enzyme Designation	Enzyme Units[a]	Substrate Designation	Substrate ml[a]	Time, min
Table II	T	11	GNA	4	10
Figure 1	T	572	GMC	0.6	Var[b]
	L	540			
Figure 2	L	54	A	0.4	10, 40
Figure 3	L	270[c]	A	0.4	Var
Figure 4	L	Var	A	0.4	40
Figure 5	T	286	A	0.1	Var
	L	540			
Figure 6	T	572[d]	B	0.1	Var
	L	540[d]			
Figure 7	T	572	GMC	0.3	Var
	L	54	A′, B′	0.1	

[a] Units, ml or μmol (GNA, GMC) per ml of enzyme reaction mixture. Variable. [c] Added at 0 and 25 min. [d] Corresponding to a relative concentration of unity.

Table II. Effect of Lyase Conditions on Transpeptidase Activity

Additives	Relative activity[d] Tris, pH 8[c]	Pyrophosphate pH 8	Tris + Pyrophosphate
None	1.00	1.46	1.30
PXP[a]	1.11	1.48	1.39
Mg^{2+b}	1.11	1.41	
PXP + $MgCl_2$		1.47	1.44
Mg^{2+}, pH 9	1.55		

[a] Pyridoxal phosphate, 0.05 m*M*. [b] $MgCl_2$ 1 m*M*. [c] 0.1 *M*. [d] 0.4 *M*.

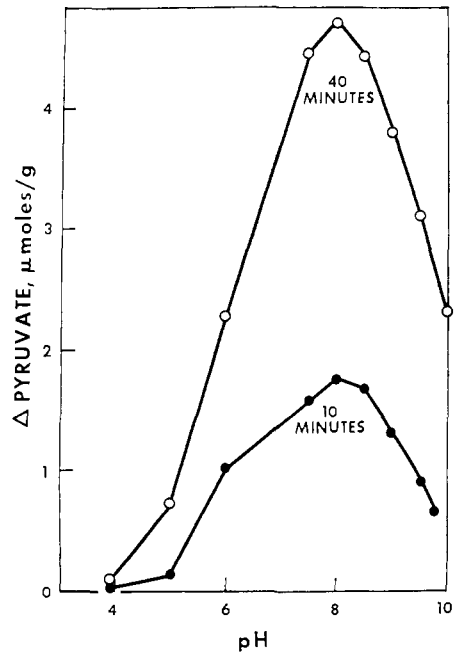

Figure 2. Effect of pH on availability to C–S lyase of substrates in onion eluate A

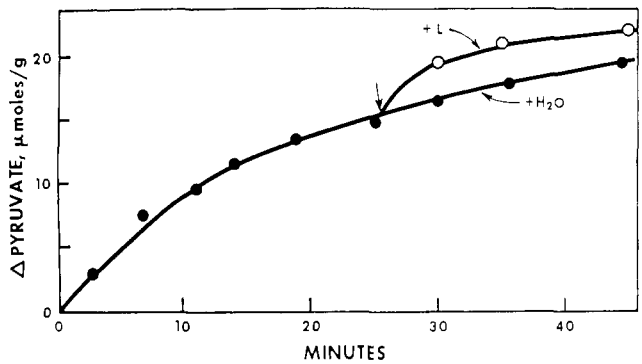

Figure 3. Time course of pyruvate production in eluate A by C–S lyase

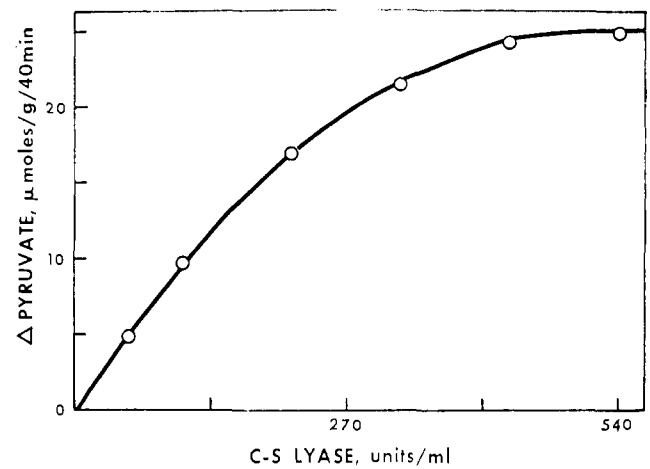

Figure 4. Effect of C–S lyase concentration on pyruvate production in eluate A

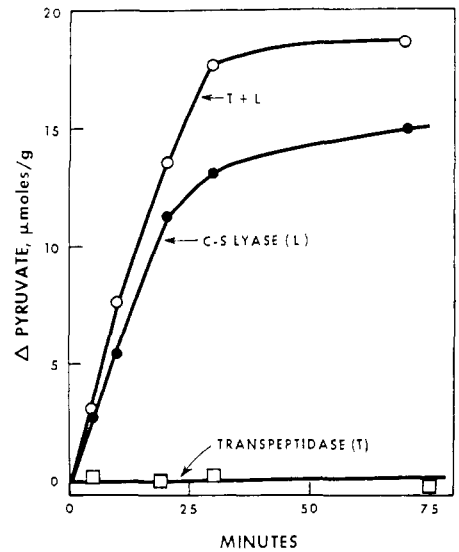

Figure 5. Coupling of C–S lyase to transpeptidase as measured by pyruvate production in eluate A

in Figures 2 to 4. Figure 2 shows the pH rate profile for the production of pyruvic acid from onion cation exchange eluate A. The optimal activity occurs at pH 8.0, about the same as for its action on *S*-ethyl-L-cysteine (Schwimmer and Kjaer, 1960). Activity is substantially absent at pH 4. At pH 6, that of onion suspensions, about half of the activity remains, thus suggesting that it may not be necessary to adjust the pH to obtain appreciable, if not optimal, enzyme action. The finding that the rate of pyruvic acid production in 40 min is somewhat less than in 10 min indicates that the rate is not proportional to the time, as shown more fully in Figure 3. There appears to be a progressive diminution in rate. Further addition of enzyme after 25 min resulted in a small but substantial increase in pyruvic acid production. This suggests that the diminution in rate is due to the accumulation of inhibitors rather than to progressive enzyme inactivation.

However, the data of Figure 4 on the effect of C–S lyase concentration show that the rate was directly proportional to the enzyme concentration over a rather broad range. Only at high enzyme concentrations, where more than half of the substrate had reacted, was there deviation from linearity.

Although no systematic variation in eluate concentration was made, a comparison of the data of Figures 3 and 5 shows that the course of enzyme action was profoundly affected by the amount of eluate. Thus, the initial rate of pyruvic acid production with 270 C–S lyase units alone, in the presence of 0.4 ml of eluate A (Figure 3), resulted in production of about 10 μmol of pyruvic acid per 10 min, whereas in the presence of 0.1 ml of eluate A and 540 lyase units, the rate was only about half this amount (Figure 5). Furthermore, the course of enzyme action in these two instances was quite distinct, probably reflecting in part the disparate kinetic constants for the multiplicity of substrates present in the onion (Schwimmer *et al.*, 1964; Schwimmer, 1968, 1969, 1970, 1971). This indicates that the range of substrate concentration used in these studies is somewhat below enzyme saturation levels and also that the kinetic parameters for the *Albizzia* enzyme are of the same order of magnitude as those obtained with onion lyases.

Coupled Reaction with Onion Eluates as Substrates. As shown in Figure 5, transpeptidase did not catalyze the production of pyruvic acid from substrates present in onion eluate A. In the presence of C–S lyase there was an incre-

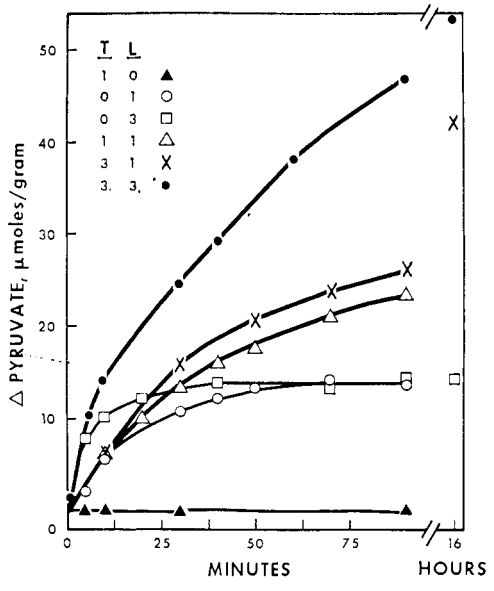

Figure 6. Coupling of C–S lyase to transpeptidase as measured by pyruvate production in eluate B. See Table I

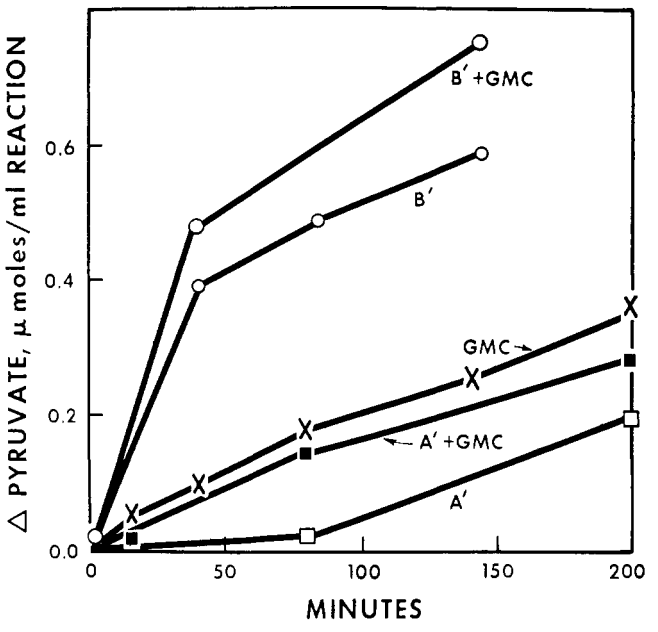

Figure 7. Coupling reaction in onion fractions A′ and B′ in the presence and absence of GMC

ment of pyruvic acid produced by the kidney enzyme over and above that formed in the presence of the *Albizzia* enzyme alone. If this increment of 4 μmol/g does indeed reflect the presence of γ-L-glutamyl peptides of S-substituted cysteine derivatives, then the concentration of the latter in this particular sample of dehydrated onion is only a fraction of that reported to be present in a fresh onion variety commonly used for dehydration (Matikkala and Virtanen, 1967). This discrepancy could be due to: (1) insufficient time and enzyme; (2) varietal differences; (3) destruction of the peptides as such during processing; and (4) losses occurring during storage. That each factor may have played a role is evidenced by a comparison of the results shown in Figure 5 with those of Figure 6. The sample used in Figure 5 was part of a large lot of onions which had been opened frequently and stored without precautions over a period of at least 10 years and had a rather stale, although strong, burnt odor upon rehydration. By contrast, onion eluate B (Figure 6) was a freshly opened premium quality powder which, upon rehydration, gave a pungent fresh onion odor. The data of Figure 6 show that, as with eluate A, the transpeptidase alone did not result in the release of pyruvic acid, whereas 540 C–S lyase units alone did produce about 13 μmol of pyruvic acid per g within 90 min, and no more was produced overnight. Tripling the C–S lyase content increased the initial rate but not the extent of product. Adding 572 transpeptidase units to 540 lyase units resulted in a 60% increase in final pyruvate levels without affecting the initial rate. Further tripling of the transpeptidase level led to only a slight increase in product in 90 min, but after 16 hr the level of product had almost tripled. The most rapid rate and highest levels of pyruvate produced, consonant with the levels of peptides reported to be present in onions (Matikkala and Virtanen, 1967), were obtained by the concomitant addition of triple quantities of both transpeptidase and lyase, resulting in a fourfold increase in pyruvic acid levels within 2 hr.

An experiment in which both enzymes were added to water extracts of onion samples A′ and B′ in the presence and absence of the peptide GMC is shown in Figure 7. Presumably most of the pyruvic acid produced was due to the coupled reaction since the endogenous lyase was not destroyed by boiling as in the case of eluates A and B. In accordance with the previous results, pyruvic acid production was much greater with sample B′ than with sample A′. The effect of B′ on product formation from GMC is about what one would expect from the simultaneous action of an enzyme on more than one substrate at relatively low substrate levels (Schwimmer *et al.*, 1964). In contrast, the addition of A′ resulted in inhibition of the coupled GMC system, thus suggesting the presence of inhibitors in A′, or that one or both enzymes have a much greater affinity for the substrates present in sample A′ than for GMC.

The foregoing results present evidence for the liberation of the precursors to onion flavor from γ-L-glutamyl peptides present in onion. It is hoped that this investigation may be of some value in further developmental investigations designed to improve the flavor of onions and onion-containing foods *via* enzyme adjuncts.

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