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Purification and Characterization of Alliinase from Garlic of Korean Origin

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한국산 마늘 alliinase의 분리 및 특성

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Abstract

마늘의 alliinase를 ammonium sulfate 분획과 hydroxylapatite chromatography, concanavalin A-Sepharose affinity chromatography에 의해 정제하여, 23%의 회수율과 7.6배 정제도를 나타내었다(specific activity 116.6 units/mg). SDS-polyacrylamide gel electrophoresis에서 단일 band를 나타내므로 순수한 alliinase로 추측되며 이 효소의 분자량은 42K로 추정된다. 기질로서 S-ethyl-L-cysteine sulfoxide를 사용한 이 효소의 V_{max}값은 2.27 μmonl/mg.min이고 K_m은 10 mM이다. 정제효소의 optimum pH는 6.5 phosphate buffer이며, 40℃에서 최대활성을 나타내었다. Activation energy value(E^*)는 4.6 Kcal/mole로 추정된다.

Key words: garlic, alliinase, purification

I. Introduction

Garlic(*Alliium sativum L.*) has had a long and interesting history of use as vegetable and spice and as home remedies in the treatment of illnesses. Cavallito and Bailey isolated a coloress oil from crushed garlic and named it allicin[1] and suggested the structure[2]. Stoll and Seebeck[3] showed that allicin is produced from alliin following damage to garlic tissue through the action of an enzyme, alliinase. This enzyme alliin alkenyl sulfenate lyase(EC 4.4.1.4) is know trivially as alliin lyase or alliinase. Alliin has no pungent odor, while allicin is pungent and unstable flavor component, which non-enzymatically decomposes into unpleasant odor products such as allyl sulfide, diallyldisulfide and diallytrisulfide[4-6].

Alliinase is found in most all of the Allium species as well as being present in some-related genera of the *Liliaseae* such as *Ipheion* and *Thulbaghia*[7]. Alliinase from *Brassica* species[8] and from the seeds of *Albizzia lophasntha*[9] have been reported. Alliinase-like enzyme have also been reported in *Pseudomonas cerecivisiae*[10] and *Bacillus subtilis*[4].

The enzymes from onion, alliium cepa have been studied in some detail[11-14]. The activity of enzyme in garlic with a crude cell-free fraction was first observed by Stoll and Seebeck[3]. Late Mazelis[15] partially purified the enzyme by Sephadex G200. Recently, Nock[16] reported direct comparison of the physical properties of alliinase of garlic and onion.

Pickled garlic, which has been cherished as one of favorite traditional Korean foods, is known to be devoid of pungent odor of fresh garlic. Kim[17] observed that there was a good correlationship between amount of allicin during tissue disruption. However, there is no report concerning factors responsible for the pungency decrease in pickled garlic of Korea.

This research was undertaken to obtain basic data concerning the properties of alliinase purified from fresh garlic of Korean origin which is one of factors related with the pungency.

II. Materials and Methods

1. Materials

Concanavalin A-Sepharose(Con A-Sepharose), hydroxylapatite, phenylmethyl sulfonyl fluoride(PMSF), S-ethyl-L-cysteine, 5'-pyridoxalphosphate, polyvinyl polypyrolidone(PVPP), acrylamide bisacrylamide, N,N,N',N'-tetramethylethylenediamine, ovalbumine, bovine serum albumine, α-methyl-D-mannopyranoside were obtained from Sigma Chemical Co. Garlic(six cloves of a garlic bulb) was purchased from local market in Taejon. All other reagents were of analytical grade.

Table 1. Summary of the purification steps of alliinase from garlic homogenate

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg.protein)	Recovery (%)	Purification (fold)
1. Homogenate	116.3	1776	15.3	100	1.0
2. $(NH_4)_2SO_4$ fractionation	68.1	1362	20.0	77	1.3
3. Dialysis	60.5	1289	21.3	73	1.4
4. Hydroxylapatite	18.4	612	33.3	35	2.2
5. Con A-Sepharose	3.5	408	116.6	23	7.6

2. Preparation of purified alliinase

Preparation of purified alliinase was carried out according to Nock and Mazelis(16). 250 g of peeled and chopped garlic cloves was blended for 1 min in a cold waring blender in 0.02M of phosphate buffer(0H 7.0) containing 10% glycerol, 1 mM PMSF, 5 mM ethylendiaminetetraacetic acid disodium salt(EDTA), 5% NaCl, 5% PVPP and 0.05% 2-mercaptoethanol. The homogenate was filtered through two layers of cheese cloth and the filtrate centrifuged at 16,300×g for 1 hr at 4℃. The supernanant solution was brought to 35% saturation with $(NH_4)_2SO_4$ and stirred slowly for 30 min at 4℃. The slurry was centrifuged at 16,300×g for 30 min at 4℃. The pellet was resuspended in approximately 100 ml of 0.05M phosphate buffer(pH 7.0) containing 10% glycerol, 1 mM PMSF and 5 mM EDTA and then dialyzed against the same buffer. The dialyzate was centrifuged at 12,000×g to remove insoluble materials. The supernatant solution was then placed in fast flow hydroxylapatite column(2.2×50 cm) and washed with 0.05M phosphate buffer to remove inactive material. The active peak was eluted using 3 volumes of 0.3M buffer. Tubes containing the enzyme activity were combined, concentrated by ultrafiltration, and then dialyzed. The concentrate was placed on a Concanavalin-A-Sepharose 4B column(20 ml). Unbounded inactive protein was eluted using a gradient of 0 to 100 mM methyl-α-D-mannopyranoside in the phosphate buffer. The amount of protein was determined by Lowry's method(18). The standard enzyme assay mixture consisted of the following in a final volume of 1 ml: 100 mM phosphate buffer(pH 6.5), 0.025 mM pyridoxal-5'-phosphate and 40 mM S-ethyl-L-cysteine sulfoxide. The above enzyme reaction mixture was incubated at 23℃ for 5 min, and then terminated by adding of 2 ml of 10%(w/v) trichloroacetic acid. Aliquots of this final solution were assayed for pyruvate by the total keto acid method of Friedemann and Haugen(19) using sodium pyruvate for calibration. One unit of enzyme activity was defined as that which produced 1 μmole of pyruvate 1 min at 23℃.

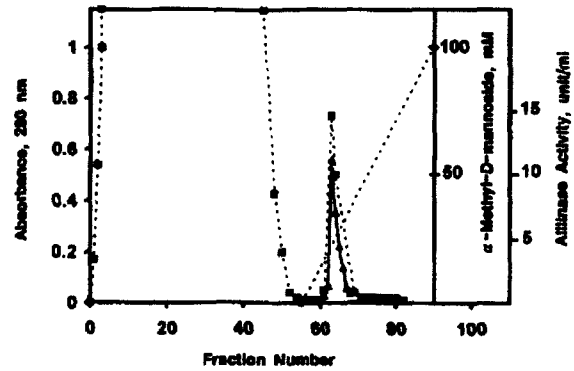

Fig. 1. Affinity chromatography of enzyme on Concanavalin-A-Sepharose column.
- Absorbance
- Alliinase activity
- α-Methyl-D-Mannoside

3. Synthesis of S-ethyl-L-cysteine sulfoxide

S-Ethyl-L-cysteine sulfoxide was synthesized from S-ethyl-L-cysteine as described by Stoll and Seebeck[20]. S-Ethyl-L-cysteine(500 mg) was dissolved in acetic acid (8 ml) under stirring and after cooling down to 10℃, 0.3 ml of hydrogen peroxide(36%) was added slowly to the solution. Then, stirring was continued for 24 hr, and the solvent was evaporated at 40℃ under rotary evaporator(Büchi, Swiss). The dry white residue was dissolved in 5 ml of distilled water and then 10 ml of hot acetone(50℃) was added to the mixture. While cooling down to room temperature, long white crystals precipitated. The crystal was used as a substrate in this experiment.

4. Protein determination

Protein was determined quantitatively by the procedure of Lowry *et al.*[18] using bovine serum albumin as standard.

5. Electrophoretic analysis of protein samples

Protein samples were analyzed using dissociating

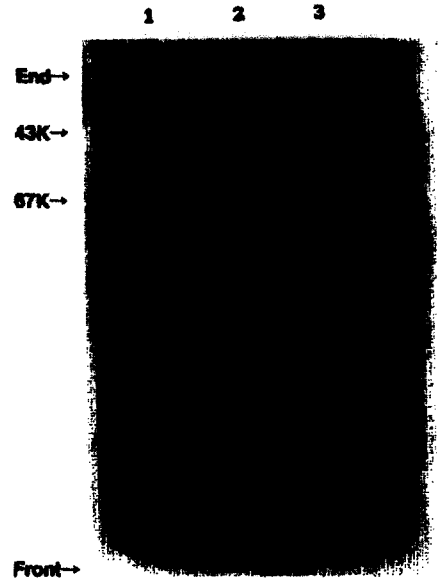

Fig. 2. SDS-polyacrylamide gel(12.5%) electrophoresis of purified alliinase(20 μg) from garlic.
Lane 1: Standard proteins(67K: bovine serum albumin, 43K: ovalbumin).Lane 2: Fraction of partially purified alliinase from hydroxylapatite.Lane 3: Fraction of purified alliinase from Concanavalin-A-Sepharose.

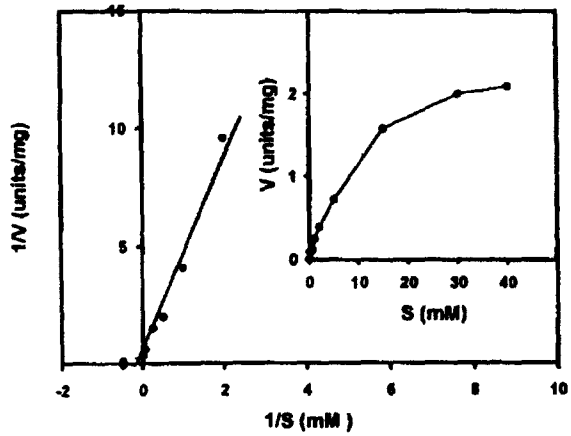

Fig. 3. Lineweaver-Burk plot for the purified alliinase.
Insert: plot of alliinase activity aganist concentrations of S-ethyl-L-cysteine sulfoxide.

SDS-PAGE buffer system(LKB, Sweden). The gels were prepared by the methods described by Hames[21] with the addition of 7M urea to the stacking and resolving gels and the sample buffers. Following electrophoresis, proteins were visualized by staining with Coomassie blue.

III. Results and Discussion

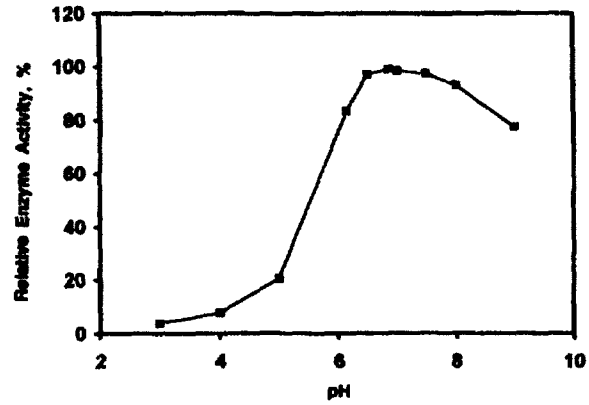

Fig. 4. Effect of pH on the activity of alliinase.
pH 3~5: 0.1M acetate buffer
pH 6~8: 0.1M phosphate buffer
pH 9: 0.1M borate buffer

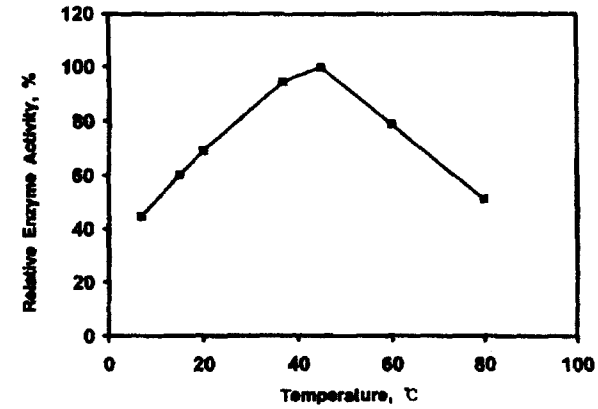

Fig. 5. Effect of temperature on the activity of alliinase.

1. Purification of alliinase

A typical purification procedure is summarized in Table 1. All of the steps were carried out at 4℃. Total amount of protein in the homogenate was 116.3 mg/100 g garlic and specific activity was 15.3 units/mg protein. Ammonium sulfate fractionation gave 1.3 fold increase in purification fold and the recovery was 77%. The hydroxylapatite chromatography gave some improvement in purification fold(1.8 fold). The final purification of alliinase was carried out using Concanavalin A Sepharose 4B affinity chromatography. As shown in Fig. 1, after washing out unbound inactive protein, the enzyme was eluted using a gradient of 0 to 100 mM α-methyl-D-mannopyrannoside in the phosphate buffer (flow rate: 0.6 ml/min, amount of fraction volume: 3 ml). Active fractions(fraction NO. 62-70) were combined.

The combined active fraction(fraction NO. 62-70) gave a 7.6-folds purification with a specific activity of

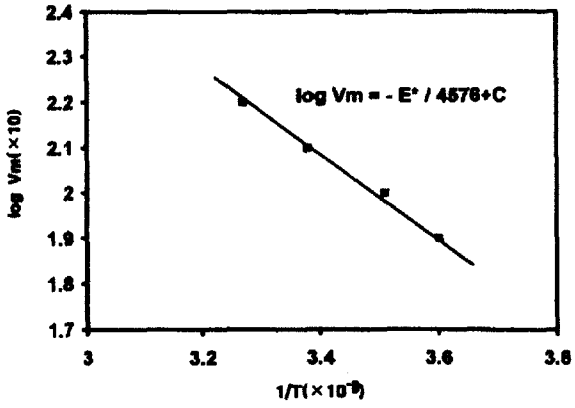

Fig. 6. The rate of hydrolysis of alliinase in 0.1M phosphate buffer pH 7.0 as a function of temperature.

116.6 units/mg and a recovery of 23%. This result was similar to that of Nock and Mazelis[16], but total protein amount in garlic of Korean origin was less one tenth than that of Nock and Mazelis[16].

2. Gel electrophoresis analysis

Twenty μg sample of purified alliinase(Table 1, fraction 5) and standard proteins were concommitantly subjected to SDS-PAGE using 12.5% gels(Fig. 2). Gels were stained with Coomassie blue. Only one band was visualized. After comparing the positions of the purified enzyme to those of standard proteins, a molecular weight of 42 kilodaltons(KD) was obtained.

3. Kinetic properties

The Michaelis-Menten constant(Km) of the purified alliinase was measured at standard assay condition except that substrate concentration ranged from 0.1 to 40 mM. For convenience, S-ethyl-L-cysteine sulfoxide was used as a substrate for assay purposes. As shown in Fig. 3 Insert, the dependency of rate on the substrate concentration showed a pseudo zero order kinetics. The apparent Km value for S-ethyl-L-cysteine sulfoxide was 10 mM and the V_{max} value was 2.27 μmole/mg.min(Fig. 3). These values were similar to those reported previously by Mazelis and Crews[15].

4. Optimum pH

The optimum pH of purified alliinase was examined over pH 3.0 to 9.0. The enzyme showed a maximal activity at pH 6.7 and its activity was very low below pH 5.0, as shown in Fig. 4. A similar pH profile was previously reported by Mazelis and Crews[15].

5. Optimum temperature

The effect of temperature on activity of the enzyme was investigated at various temperature ranging from 7 to 80℃. As shown in Fig. 6, the enzyme exhibited optimum activity at 40℃. To estimate activation energy of the alliinase (Fig. 7), a replot of log velocity versus reciprocal absolute temperature was attempted. From the slope obtained, the activation energy of the enzyme was calculated by Arrhenius equation[22], the activation energy of the enzyme was estimated to be 4.6 Kcal /mole.

IV. Conclusion

Alliinase from garlic of Korean origin was purified by ammonium sulfate fractionation, hydroxylapatite chromatography and finally, Concanavalin A-Sepharose affinity chromatography, which gave a 7.6-folds purification(specific activity, 116.6 units/mg) with a recovery of 23%. SDS-polyacrylamide gel electrophoresis showed a single major band, suggestive of a relatively pure alliinase, and the M.W. of the enzyme was estimated to be 42K. The enzyme gave a V_{max} value of 2.27 μmole/mg.min and a K_m value of 10 mM for S-ethyl-L-cysteine sulfoxide used as a substrate. The optimum pH of purified enzyme was 6.5, phosphate buffer. The optimum pH of purified enzyme was 6.5, phosphate buffer. The enzyme exhibited a maximal activity at 40℃, and the activation energy value(E^*) was estimated to be 4.6 Kcal/mole.

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