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Purification and Characterization of Tyrosinase from *Solanum melongena*

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Abstract : Tyrosinase was purified from *Solanum melongena* by ammonium sulfate precipitation, Sephadex G-150 and DEAE-Sephacel column chromatography. The molecular weight of the purified tyrosinase was approximately 88,600 daltons with 805 amino acid residues. The amino acid composition showed the characteristic high contents of glycine, glutamic acid and serine residues. The enzyme had high substrate specificity towards (+)-catechin. The K_m value for L-DOPA was 20.8 mM. L-ascorbic acid, β-mercaptoethanol, sodium diethyldithiocabamate, KCN and NaN_3 had strong inhibitory effects on enzyme activity. Sodium diethyldithiocabamate was a competitive inhibitor of the enzyme with a K_i value of 5.2×10^{-2} mM. The optimum pH of the enzyme was 9.0 and the optimum temperature was 65°C with L-DOPA as a substrate. In addition, the activity was enhanced by addition of Ca^{2+} or Cu^{2+}, but decreased in the presence of Fe^{2+}, Fe^{3+} and Zn^{2+} ions.

Key words : amino acid composition, eggplant, enzymatic characteristics, *Solanum melongena*, tyrosinase.

Tyrosinase (EC. 1.14.18.1) is a copper-containing monooxygenase that is responsible for melanin biosynthesis (Mayer, 1987). It is a bifunctional enzyme catalyzing both the hydroxylation reaction that converts tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and the oxidation reaction that converts DOPA into dopaquinone.

Tyrosinase activities are widely distributed from mammals to microorganisms. Especially in mammals, tyrosinases have been purified, and their properties and functions in cells have been studied extensively (Riley, 1993). Tyrosinase activities have also been found in plant, and plays important roles in plant metabolism. These enzymes are known to be involved in respiration system, intermediary metabolism, regulation of oxidation-reduction potential, antibiotic effect and wound system (Kevin *et al.*, 1988).

Tyrosinases are also related to the enzymatic browning of fresh and processed fruits and vegetables, and have been of concern in the food industry (Paulson *et al.*, 1980). Thus, tyrosinases have been purified and studied from numerous fruits and vegetables for many years. The enzymes from *Agaricus bisporus* (Strothkamp *et al.*, 1976), and *Neurospora crassa* (Lerch, 1983) have been investigated most extensively from both a structural and functional point of view. However relative activities, optimum pHs of activity and molecular weights of these enzymes show considerable variations (Mayer, 1987). Despite these efforts, little is known about purification and properties of tyrosinase from *Solanum melongena* (eggplant).

This study was undertaken to describe the purification and enzymatic and structural properties of this enzyme, to characterize the factors that affect its activity and to compare its properties with those of enzymes from other sources.

Materials and Methods

Materials

The *Solanum melongena* used in this study, was supplied from Aha San, Korea. L-DOPA, SDS-PAGE molecular weight marker, Coomassie Brillient Blue R250, DEAE-Sephacel and glutathione were purchased from Sigma (St. Louis, USA). Sepadex G-150 was purchased from Pharmacia (Uppsala, Sweden). Sodium diethyldithiocarbamate was obtained from Merk (Darmstade, Germany). All the reagent used were of reagent grade unless otherwise stated.

Purification of enzyme

The *Solanum melongena* was homogenized 5 times with 100 mM potassium phosphate buffer (pH 6.7) in a waring blender for 1 min. The mixture was centrifuged at 20,000×*g* for 30 min and gave crude ex-

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tracts of 1200 ml. A 25~70% $(NH_4)_2SO_4$ precipitate was obtained from crude extracts and suspended into buffer A (20 mM potassium phosphate buffer/1 mM EDTA, pH 6.7). This solution was dialyzed 3 times every 8 h against buffer A. The dialyzed solution was concentrated using a PM-10 membrane (Amicon) and loaded on a Sepadex G-150 column. The fractions with tyrosinase activity were pooled and then applied to a DEAE-Sephacel column (25×250 mm) equilibrated with buffer A. After washing the column with buffer A, bound proteins were eluted with a linear gradient of NaCl (0~500 mM) in buffer A at 0.4 ml/min. The active fractions were pooled and were dialyzed against buffer A. The dialyzed solution was applied to a DEAE-Sephacel column a second time. After washing the column with buffer A, bound proteins were eluted with a linear gradient of potassium phosphate buffer, pH 7.0 (0~500 mM). Purified enzyme was collected from the high part of activity in DEAE-Sephacel column chromatography. This collected purified enzyme was used for next experiments. Unless otherwise indicated, all purification procedures were performed either at 4℃ or on ice.

Enzyme assay

The tyrosinase activity was assayed by a procedure of Sung and Cho (1992) spectrophotometrically, measuring conversion of L-DOPA to the red colored oxidation product, dopachrome. The initial rate of the reaction is proportional to the enzyme concentration. An aliquot containing tyrosinase was incubated for 5 min at 30℃. At time zero, 1 ml of L-DOPA solution (4 mg/ml) was measured at 475 nm. After incubation for additional 5 min, the mixture was shaken again, a second reading was determined and was measured for 3 min. The change in absorbance was proportional to the enzyme concentration. One unit of enzyme corresponds to the amount that catalyzes the transformation of 1 μmol of substrate to product per min under the above condition and produces 1.35 changes in absorbance. Specific activity was expressed as enzyme units per milligram of protein. The protein content of the enzyme preparation was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out *via* the method of Laemmli (1970) in 12.5% gels. Gels were stained with Coomassie Brilliant Blue or with the thymol-sulfuric acid as described by Rauchsen (1990). For determination of molecular mass, the standard proteins used were phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and lysozyme (14.4 kDa).

Amino acid analysis

Analysis of amino acids was divided into analysis of general, cysteine and tryptophan. Sample was dissolved in 200 μl distilled water and then dried to 18 μl each after filtering. The dried sample was hydrolyzed at 110℃ for 24 h with 6 N HCl. Cysteine analysis was carried out after HCl hydrolysis and peroxidation and tryptophan analysis was carried out after alkaline hydrolysis. Amino acid hydrolyzates were derivatized to phenylisothiocyanates and dissolved to a volume of 200 μl after the sample was dried completely. After microcentrifugation, the supernatant was diluted with 100 μl and put in the autosampler of HPLC.

pH stability

Enzyme stability at various pH values was determined by using the following buffers (100 mM) at the indicated pH: citrate-sodium phosphate buffer, from 4.0 to 6.0; potassium phosphate buffer, from 6.0 to 7.5; Tris-HCl buffer, from 7.5 to 9.5; carbonate-bicarbonate buffer, from 9.5 to 10.5. No change in the pH was noted during the 20 h storage period at 4℃. At the end of the storage period, the remaining activity was determined.

Heat stability

The enzyme was incubated at each temperature for 10 min at a protein concentration of 100 μg/ml and the remaining activity was determined.

Results and Discussion

Purification of tyrosinase

The results of purification of the enzyme from *Solanum melongena* are summarized in Table 1. The solanum melongena was homogenized in 20 mM potassium phosphate buffer containing 5 mM polyethylene glycol and was centrifuged. About 70% of the total enzyme activity in the crude extract was precipitated between 25 to 70% ammonium sulfate saturation. Further activity, however, was lost during the dialysis to give an overall yield of 42% in this step. This loss was due to aggregation of the enzyme caused by the quinones produced by the enzyme in the presence of endogenous phenolic compounds (Butt, 1980). Sephadex G-150 was used to remove some small molecular weight proteins including phenolics from the dissolved ammonium sulfate pellet. Upon chromatography on Sephadex G-150 of the ammonium sulfate fraction, the activity was eluted in a broad peak (data

Table 1. Purification of tyrosinase from *Solanum melongena*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
1. Crude extract	889	1594.1	0.56	1.0	100
2. Ammonium sulfate[a]	370	333.0	1.11	2.0	42
3. Sephadex G-150	223	61.3	3.64	6.5	25.1
4. DEAE-Sephacel I	104	5.0	20.8	37.1	11.8
5. DEAE-Sephacel II	36	0.6	60.0	107	4.0

[a] About 70% of the total enzyme activity in the crude extract was precipitated between 25 to 70% ammonium sulfate saturation. Further activity, however, was lost during the dialysis to given an overall yield of 42% in this step.

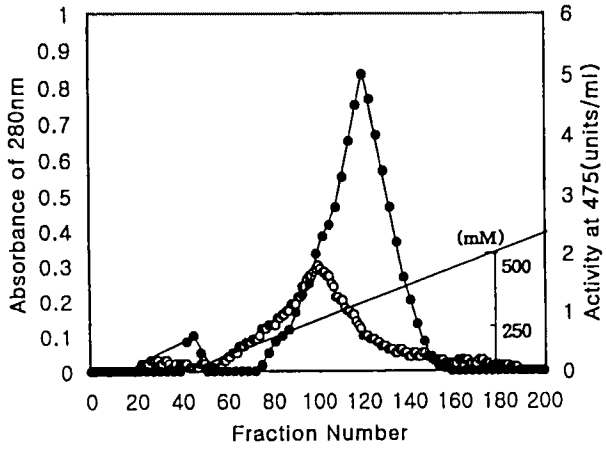

Fig. 1. Elution profile on DEAE-Sephacel column chromatography (25×250 mm). An aliquot of each fraction was assayed for protein (○—○) and activity (●—●). A linear gradient indicated b y the solid line.; 0~500 mM NaCl.

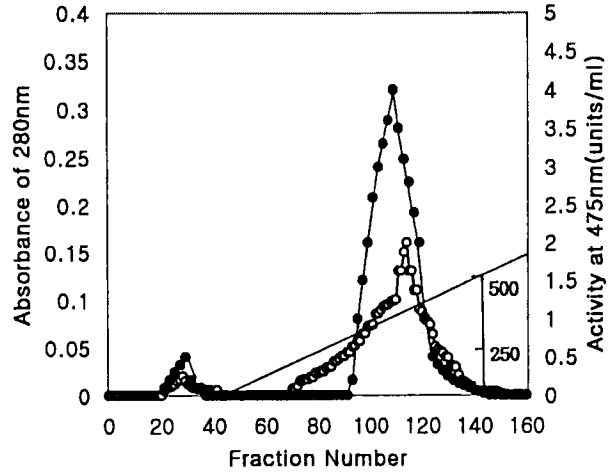

Fig. 2. Elution profile on DEAE-Sephacel column chromatography (25×250 mm). An aliquot of each fraction was assayed for protein (○—○) and activity (●—●). A linear gradient indicated by the solid line.; 0~500 mM potassium phosphate buffer.

not shown). When this fraction was passed through a DEAE-Sephacel column and eluted with a linear gradient of NaCl, the activity was concentrated into a peak, as shown in Fig. 1. The active fractions were re-chromatographed on DEAE-Sephacel and then eluted with a linear gradient of potassium phosphate buffer (0~500 mM). Fig. 2 shows the chromatogram thus obtained. Specific activity of tyrosinase was increased from 0.56 U/mg to 60.0 U/mg when L-DOPA was used. The result represents an 107-fold purification. A similar result was obtained from a study of purified tyrosinase from potato tuber, indicating a 132-fold purification (Kwon and Kim, 1996). In *Solanum melongena*, we found one peak for tyrosinase activity used upon chromatography on DEAE-Sephacel. Raymond *et al.* (1993) also reported the purification from sunflower seeds of one form of enzyme by DEAE-cellulose column chromatography. On the other hand, the multiplicity of tyrosinase has been found in the clingstone peach (Wong *et al.*, 1971) and mushroom (Gerritsen *et al.*, 1994) by DEAE-cellulose column chromatography and hydroxylapatite column chromatography, respectively. This difference is probably due to the difference in isolation methods.

Structural properties

The enzyme gave a single band on electrophoresis in the presence of SDS, as shown in Fig. 3. Comparison of relative mobility of the purified tyrosinase by SDS-PAGE with a standard protein indicated that molecular weight of the enzyme was approximately 88.6 kDa, whereas G-150 gel filtration gave approximately 90 kDa. The crude and purified enzymes appeared as a single band on SDS-PAGE when stained for activity with L-DOPA (Fig. 3, lane 3, 4). The enzyme could not be revealed on SDS-PAGE gels by staining with the thymol-sulfuric acid, which indicates that it is a non-glycosylated protein. The molecular weights of the plant enzymes have been shown to be approximately 40~70 kDa in sunflower seed (Raymond *et al.*, 1993), potato tuber (Kwon and Kim, 1996) and plum (Siddiq *et al.*, 1996) etc., and are lower than that of the *Solanum melongena* enzyme.

The amino acid composition of the enzyme is reported in Table 2 together with that of persimmon tyro-

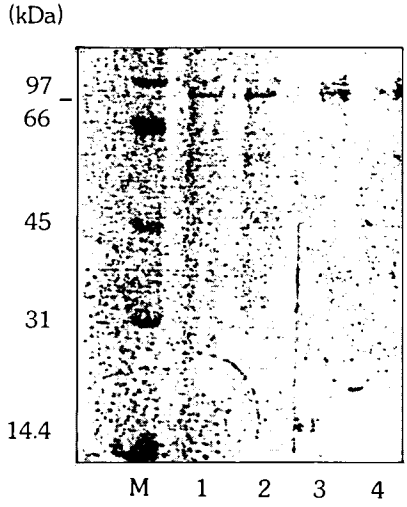

Fig. 3. SDS-PAGE of purified solanum melongena tyrosinase. Lane 1 and 2, purified enzyme stained for protein; lane 3, crude enzyme stained for activity with 10 mM L-DOPA; lane 4, purified enzyme stained for activity with 10 mM L-DOPA; lane M, molecular weight standard marker.

sinase (Sung and Cho, 1992). The amino acid composition of the enzyme was generally similar to that of the persimmon tyrosinase; both have high contents of glycine, glutamic acid and aspartic acid residues. The difference between the *Solanum melongena* and persimmon enzyme was notable in the content of histidine and serine residues on molar percent basis. The *Solanum melongena* enzyme also has a low content of methionine and cysteine residues. The relatively low content of sulphur amino acids has been reported in tyrosinases from broad bean (Robb *et al.*, 1965), potato tubers (Balasingam and Ferdinand, 1970) and grapes (Kidron *et al.*, 1977).

Enzyme kinetics

The effect of L-DOPA concentration on tyrosinase activity was determined at 30°C. L-DOPA was prepared in 100 mM potassium phosphate buffer, pH 6.7. Michaelis-Menten constant and the maximum velocity of the enzyme were determined by Lineweaver and Burk plot. The K_m value for the enzyme was 20.8 mM. The K_m value was 4.2~7.1 mM in the case of apple tyrosinase (Janovitz-Klapp *et al.*, 1990), 11.8 mM from persimmon (Sung and Cho, 1992) and 9.0 mM from monastreel grape (Alvaro *et al.*, 1988). This indicates that solanum melongena tyrosinase has a weaker affinity for L-DOPA than apple, persimmon and monastreel grape tyrosinases.

Substrate specificity

The substrate specificity of the enzyme toward various compounds is shown in Table 3. A number of monophenols, dihydroxyphenols and trihydroxyphenols were used to determine the substrate specificity of the enzyme. L-DOPA was used as the standard Substrate. The enzyme showed high activity toward (+)-catechin, catechol, (±)-catechin, resorcinol and pyrogallol. On the other hand, activity was not observed with 4-hydroxy-3-methoxycinnamic acid and 4-hydroxymethoxybenzoic acid. The number of hydroxyl groups and the position in the benzene ring of the substrate affected the oxidase activity (Park and Luh, 1985). The substrate specificities of the *Solanum melongena* tyrosinase were moderately similar to those of enzymes of persimmon (Sung and Cho, 1992) and plum (Siddiq *et al.*, 1996). The enzyme catalysed both the hydroxylation of monophenols and the oxidation of diphenols and triphenols, although it had very low activities towards monophenols. Similar results were obtained from studies of tyrosinase from broad bean (Zenin and Park, 1978), kiwi fruit (Park and Luh, 1985), persimmon (Sung and Cho, 1992) and potato tuber (Kwon and Kim, 1996). On the other hand, many tyrosinases lack monophenol activity (Benjamin and Montgomery, 1973; Anosike and Ayaebene, 1981;

Table 2. Amino acid composition of tyrosinase from *Solanum melongena* compared to that of persimmon

Amino acid	*Solanum melongena* tyrosinase (Residues/Molecule)[a]	Persimmon tyrosinase (Residues/Molecule)[b]
CYA[c]	6.0	18.6
ASP	60.2	27.9
GLU	114.1	21.8
SER	112.0	13.3
GLY	145.5	27.9
HIS	19.0	1.3
ARG	7.5	7.8
THR	31.3	15.4
ALA	80.6	17.3
PRO	29.8	16.7
TYP	13.2	5.9
VAL	41.9	10.6
MET	1.9	2.0
ILE	29.4	6.7
LEU	39.1	10.3
PHE	16.2	9.7
TRP	29.7	4.7
LYS	27.9	9.1
Total	805	227

[a] Calculated assuming a molecular weight of 88,600 for the enzyme, as found in this study. The final values are averages unless otherwise specified.
[b] Sung and Cho, 1992.
[c] CYA means the sum cysteic acid and oxidized cystine.

Table 3. Substrate specificity of tyrosinase from *Solanum melongena*

Substrate	Relative activity[a]
Monohydrophenol	
vanillic acid	27
tyramine	36
p-coumaric acid	23
o-coumaric acid	9
p-hydroxybenzoic acid	23
4-hydroxy-3-methoxycinnamic acid	0
4-hydroxy-methoxybenzoic acid	0
Trihydroxyphenols	
pyrogallol	120
phloroglucin	73
Dihydroxyphenols	
L-DOPA	100
catechol	127
3,4-dihydroxybenzoic acid	86
caffeic acid	55
chlorogenic acid	46
epicatechin	91
(+)-catechin	164
(±)-catechin	127
orcin	46
resorcinol	132

[a] Relative absorbance values were indicated showing the absorbances at 475 nm with L-DOPA as 100%.

Raymond *et al.*, 1993). This difference may also reflect the difference in organs and species.

Effect of inhibitors

Several compounds were tested for tyrosinase inhibitory action. The effect of inhibitors on the enzyme activity is shown in Table 4. Enzyme activity was totally inhibited by addition of 10 mM of L-ascorbic acid. Sodium diethyldithiocarbamate and β-mercaptoethanol also showed the same inhibitory effect on tyrosinase activity. Luh and Phithakpol (1972) showed that sodium diethyldithiocarbamate caused temporary decolorization of the solution, with no evidence as to made of action, the complexing reaction may have withdrawn the copper ion from the tyrosinase nucleus, inhibiting the enzyme. For inhibitor effect of the enzyme, sodium diethyldithiocarbamate was used at concentration ranged from 0.045 mM to 0.065 mM and L-DOPA was used at concentration ranged from 10 mM to 20 mM. Enzyme activity was determined at 30°C. Michaelis-Menten constant of the enzyme was determined by the Lineweaver and Burk plot. The results indicated that sodium diethyldithiocarbamate was a competitive inhibitor of the enzyme with the K_i value of 5.2×10^{-2} mM. Duckworth and Coleman (1970) showed, using

Table 4. Effect of inhibitors on tyrosinase from *Solanum melongena*

Inhibitor	Concentration (mM)	Relative inhibition (%)
L-Ascorbic acid	10	100
	1	12
Sodium chloride	10	69
	1	29
EDTA	10	54
	1	39
β-Mercaptoethanol	10	100
	1	100
Gluthathione	10	39
	1	36
L-Cysteine	10	36
	1	26
Sodium diethyldithiocabamate	10	100
	1	100
Cinnamic acid	10	85
	1	54
Thiourea	10	69
	1	42
Benzoic acid	10	43
	1	34
Sodium azide	10	91
	1	46
Potassium cyanide	10	92
	1	60
Phenylalanine	10	48
	1	20
8-hydroquinoline	10	66
	1	2
L-Trytophan	10	39
	1	38

mushroom catechol oxidase, that cyanide was competitive to oxygen while benzoic acid competed with phenolic substrate.

Effect of metal ions

The effect of metal ions on tyrosinase activity is shown in Table 5. The concentration of various metal ions used in this experiment was 2 mM. Enzyme activity was assayed at 30°C. Enzyme activity was increased in the presence of Ca^{2+}, Cu^{2+} and Mg^{2+} ions, but decreased by addition of K^{+}, Fe^{2+}, Fe^{3+} and Zn^{2+} ions.

Effects of pH and temperature

The effect of pH on L-DOPA oxidation by the enzyme is shown in Fig. 4. The optimum activity of the enzyme on L-DOPA was observed in the range of pH 8.5 ~9.5. In the range of pH 5~7.5, the enzyme showed 20~30% of its maximum activity. On the other hand, a pH optimum near neutrality has been observed for

Table 5. Effect of various metal ions on tyrosinase from *Solanum melongena*

Ions	Relative activity (%)	Ions	Relative activity (%)
None	100	$HgSO_4$	60
$BaCl_2$	75	KCl	10
$CaCl_2$	180	LiCl	80
$CuCl_2$	190	$MgCl_2$	130
$FeCl_2$	0	NaCl	70
$FeCl_3$	0	$ZnSO_4$	0

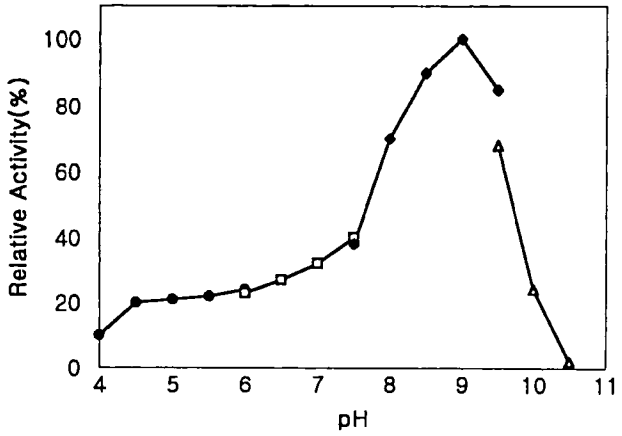

Fig. 4. Effect of pH on solanum melongena tyrosinase. The maximum activity was expressed as 100%. pH 4.0~6.0 : 100 mM citrate-200 mM sodium phosphate buffer; pH 6.0~7.5 : 100 mM potassium phosphate buffer; pH 7.5~9.5 : 100 mM tris-HCl buffer; pH 9.5~10.5 : 100 mM carbonate-bicarbonate buffer.

tyrosinase of Royal Ann chemes (Benzamin and Montgomery, 1973), pear (Halium and Montgomery, 1978) and sunflower seeds (Raymond *et al.*, 1993). Effect of pH stability on the L-DOPA oxidation by the enzyme was observed and enzyme ativity was determined at pH ranged from 4.0 to 10.5. The remaining activity after 60 min storage at each pH was measured for 10 min at 30°C. The relative activity changed at various pH stabilities and maximum activity was obtained at pH 9.0 (data not shown). On the other hand the sunflower tyrosinase remained fully active between pH 4.8 to 7.9 after 20 h exposure to buffer of different pH at 4°C (Raymond *et al.*, 1993).

In order to understand the effect of temperature on enzyme activity, the reaction mixture was incubated from 10°C to 85°C. The optimum temperature for enzyme activity was 65°C (Fig. 5), which is a higher temperature as compared to others. The optimum temperature for tyrosinase from monastrell grape was 25~40°C (Alvaro *et al.*, 1988) and from plum was 20°C (Siddiq *et al.*, 1992). Such high maximum temperature

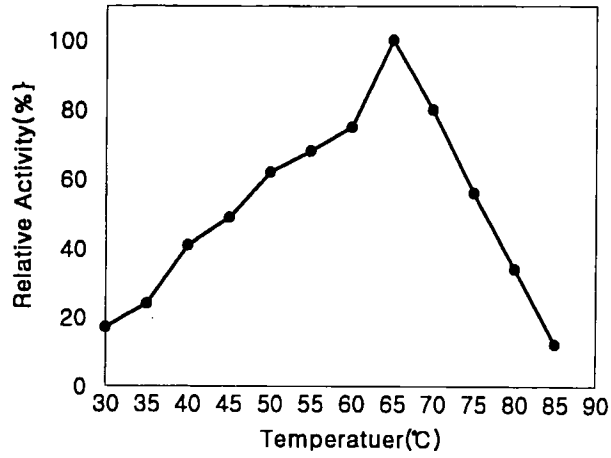

Fig. 5. Effect of temperature on *Solanum melongena* tyrosinase.

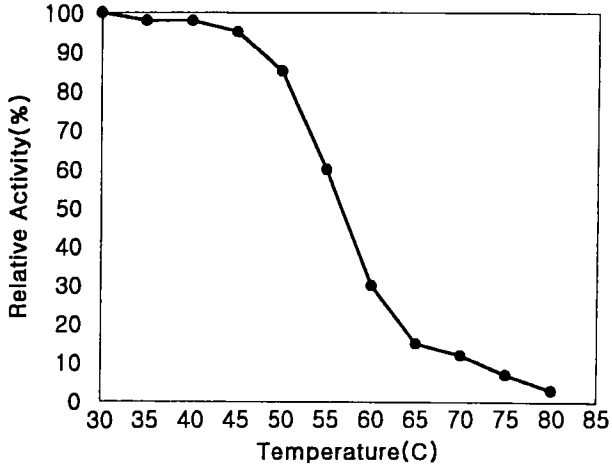

Fig. 6. Thermostability on *Solanum melongena* tyrosinase The enzyme was heated at each temperature for 10 min.

of *S. melongena* may be closely connected with its habitat. The thermostability of tyrosinase activity is shown in Fig. 6. To determine the effect of enzyme stability during heating, the reaction mixture was incubated at various temperatures ranging from 10°C to 85°C for 10 min and the remaining activity was determined. The enzyme was fairly stable to incubations at temperatures up to 45°C. Above 50°C, the tyrosinase activity declined rapidly as the temperature increased but the enzyme was not completely inactivated even at 80°C. This indicated that tyrosinase activity and thus its browning action could not be prevented at high temperature (< 80°C) treatment.

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