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Partial Purification and Some Properties of Amylases from Germinating Corn(*Zea mays* L.)

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Abstract

The purpose of this study was focused on investigation of biochemical properties of amylases in germinating corn(*Zea mays* L.) the amylase(Ⅰ), (Ⅱ) and (Ⅲ) from germinating corn seeds were partially purified by ammonium sulfate precipitation, DEAE-Sephadex A-50 ion exchange column chromatography and Sephadex G-100 gel filtration. The last step was effective for separation of the corn amylases to a homogeneous state. the purified amylase(Ⅰ) was identified as a kind of α-amylase from the fact that 5% starch solution was hydrolysed into mainly maltose and maltotetrose by it, and amylase(Ⅱ) and amylase(Ⅲ) were enzymes producing maltotetrose as main product. The molecular weight and specific activity of the amylase(Ⅰ), (Ⅱ) and (Ⅲ) were determined to be 54,000 and 70.47 unit/㎎, 39,000 and 62.98 unit/㎎, and 51,000 and 80.39 unit/㎎, respectively. It showed a tendency to increase the amylases activities in presence of Ba, Ca, Co and Fe groups, but inhibite in that of Ag, Sn, Hg and Zn groups, and amylase(Ⅰ), (Ⅱ) and (Ⅲ) remained stable at pH 5~6 and 20℃ for 40 days in containing of 1mM $CaCl_2$. The optimum pH and optimum temperatures were pH 6, pH 5 and pH 6 and 35℃, 55℃ and 55℃, respectively. These results suggest that the amylase(Ⅰ), (Ⅱ) and (Ⅲ) were different amylases.

Introduction

The amylases act on starch, glycogen and derived polysacharides to hydrolyze the α-1, 4 glucosidic linkages. The amylases may be divided into three groups, the α-amylase[EC 3.2.1.1], β-amylase[EC 3.2.1.2] and gluco amylase[EC 3.2.1.3.]. There are much of studies on amylases from microorganisms, but not so much as yet from higher plants. All the amylases are similar in molecular weight(60,000± 5,000) and inactivation by the modification of sulfhydryl group with p-chloromercuribenzoic acid(PCMB). But some physicochemical properties are different between plant-type amylase(optimum pH 5-6, pI 5-6)[1-5] and bacterial amylase(optimum pH 6-7, pI 8-9)[5-10]. Although Manners et al[11] Suggested the occurence of three of amylase in sweet corn, the experimental results were not conclusive ; 1) their amylase preparation was a crude purification by gel filtration on Sephadex G-75. 2) The optical rotation of the sugars formed by the enzyme was not tested. Therefore, we thought enzyme activity was high in the course of germination and tried to purify the enzyme in corn seeds to check what type of amylase it may be ; Some features of the amylase obtained are described in this paper.

Materials and Method

Materials

Corn seeds(*Zea mays* L.) were harvested in 1985 and were purchased from a market in jagalchyi, pusan, korea ; Sephadex G-100 and DEAE-Sephadex A-50 from sigma, USA ; All other chemicals were reagent grade and were used without further purification.

Seeds germination

Corn seeds were soaked in running water for 1 hour and germinated on incubator at 28± 1℃ for 3 days.

Amylase activity assay

The reaction mixture contained 2mℓ of 0.1M phosphate buffer, pH 6.0 containing 1mM $CaCl_2$, 0.1mℓ of enzyme solution. the reaction was started by adding 1mℓ of 1.5% soluble starch, and was incubated in water bath at 35℃ for 10min. the reaction was terminated by adding 5mℓ of 0.5M acetic acid and was measured by monitoring the increase of absorbance at 650nm. As a standard method, it was measured according to the method of Blue Value[12].

One unit of amylase activity was defined as the amount corresponding to the release of 10% per 10min, and the specific activity which is a measure of the purity of the enzyme preparation, is expressed as units per mg of material.

Protein determination

Protein content was usually determined by the method of Lowry et al.[13]. Protein concentrations of column eluated, however, were routinely monitored by the absorbance at 280nm.

Preparation of the crude enzyme extract

Usually, one thousand grams of germinated corn were homogenized in a waring blender in 1.0 liter of cold 0.1M phosphate buffer, pH 6 containing 1mM $CaCl_2$. The resulting homogenate was filtered through nylon cloth and then centrifuged for 15min at 20,000rpm. The supernatant is the crude enzyme extract used in purification experiments.

Purification procedure

The folowing steps were carrid uot at 4℃ and the buffer used throughout was 10mM potassium phosphate buffer pH 6.0, containing 1mM $CaCl_2$ and will be referred to simply as the 'buffer' unless specfied otherwise. The crude enzyme extract was fractionated with ammonium sulfate and the proteins precipitated between 30 to 80% saturation were dissolved in a small volume of the buffer. After desalting the fractions of enzymes eluted from the gel were loaded on a DEAE-Sephadex A-50 column(20×280mm) reequilibrated with the buffer at a flow rate of 24mℓ/hr and fractions of 7mℓ were collected. Active fractions were pooled and concentrated with 80% ammonium sulfate saturation. The concentrated enzyme solution was reapplied to a DEAE-Sephadex A-50 column(20×280mm). After washing with 200mℓ buffer the column was eluted(24mℓ/hr) by increasing the concentration of sodium chloride up to 0.5M and were pooled concentrated and dialyzed against the buffer. A column of 20×280mm was used and linear NaCl gradient of 0.0M-0.5M and 0.2M-0.45M were used respectively to purify F-I and F-II. After dialysis and concentration, the resulting enzyme solutions were walking the Sephadex G-100 gel chromatography(25×900mm), respectively.

Examination of the effects of various compounds on the amylase activity

The enzyme solutins containing a test compounds(1mM, 0.1mM) was pre-incubated at 20℃ for 1hr. After the pre-incubation, the remaining activity was determined by the standard blue value

method.

Action mode of purified amylase

Identification of sugars formed by the purified amylases reaction ; Sugars hydrolyzed by the amylase action and were analyzed on Bio Rad HPX-87 column using HPLC(Waters, Co., Ltd equipped with RI detector. Elution was performed with the solvent of distilled water at the flow rate of 0.7 mℓ/min, and 120kg/cm pressure.

Determination of free sugars

The contents of free sugars in the course of germination were measured by Conrad and Palmer's modified method[15-20].

Germinated seeds were homogenized in a waring blender for 5 minites. The samples were covered with sufficient 100% ethanol to make the final concentration of ethanol 80%. The sample and ethanol were then blended at high speed for 2−3 min(depending on tissue softness). The resulting slurry was refluxed under stirring for 3hr with water bath(80℃). The extract was then filtered through Whatman No. 54 paper. The residue and flat-bottom flask were washed with additional 80% etanol and refluxed with 30mℓ Hexane. The extract plus washing were then reduced to a volume less then 25mℓ by using vacuum evaporator. Samples were concentrated until the ethanol odor was completely gone. Finally concentrate was made to 10mℓ with distilled water and filtered through Sep-pak C_{18} cartrige. Sugar analysis were carried out by using HPLC(Waters) ; Injection volumn ; 10ul, mobile phase ; acetonitrile : water : ethanol(80 : 15 : 5), flow rate ; 1.8mℓ/min, attenuation ; 8. Before the injection, however, the samples were filtered through a 0.45um meterical membrane(Gelmann filtration products, Ann Arbor, MI) to further ensure removal of any particulate impurities that might be present.

Results and Discussion

The solid ammonium sulfate was gradually added to the supernatant to 30% saturation. The precipitate formed was removed by centrifugation at 5,000×g for 10min and discard. Solid ammonium sulfate was added to the supernatant to 80% saturation, followed by standing overnight at the cold room(4℃).

Purification of amylase(I), (II) and (III)

Three amylase activities were partially purified from germinating corn by ammonium sulfate fraction, first DEAE-Sephadex ion exchange column chromatography. The three peaks appeared on the DEAE-Sephadex A-50 column chromatography were pooled and resolved respectively. Each activity peak was further purified by the second DEAE-Sephadex A-50 column chromatography. Amylase(I) was eluted at NaCl concentration of 0.35M and amylase(II) at 0.4M, amylase(III) at 0.45M, and was then applied to the column(25×900m) filled with Sephadex G-100(Sigma) and eluted with the buffer at the flow rate of 10mℓ per hour which contained 880.9, 680.2, 820units of total enzyme activities and were combined. the partial purified amylase(I), (II) and (III) thus obtained. As shown were summarized the increases in the specific activity of amylases and the yield during the purification.

The low yields(Table 1) observed in this study appear to be related to the low purification fold. Because we obtained apparently homogeneous enzymes after gel chromatography, the amylases should represent about 0.016%, 0.014%, and 0.0136 % of the total proteins of corn seeds. In fact, most reported results of amylases purification show low purification folds and yields. It suggested that such enzyme inactivation might occur during isolation and handling.

Table 1. Summary of partial purification of corn amylase I, II and III

Purification	Volume (mℓ)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)
Crude extract	5,000	75,000	0.50	38,000	100
30~80$(NH_4)_2SO_4$ Fractionation & Dialysis	600	14,000	1.90	26,600	70
Chromatography on DEAE-Sephadex A-50 (F-I)	55 52 58	353.0 259.5 231.8	17.97 17.60 17.78	6,345 4,569 4,122	39.6
Concentration with 80%$(NH_4)_2SO_4$ precipitation	20 20 20	250.6 210.8 190.2	16.44 13.52 11.57	4,120 2,850 2,200	24.1
Rechromatography on DEAE-Sephadex A-50(F-II)	28 14 14	40.2 22.8 18.8	33.63 59.68 67.40	1,352 1,360 1,065	9.9
Gel filtration on Sephadex G-100	20 20 20	12.5 10.8 10.2	70.47 62.98 30.39	880.9 680.2 820	6.2

Determination of free sugars

Germinated corn sample were analyzed by using HPLC. Hydrolysis of sugar was increased highly in the course of germination. Especially, the 3 days germinated corn was consisted of DP_4(210.6mg%), DP_3(little), maltose(400.8mg%), fructose(112.8mg%) and glucose(313.2mg%). Therefore, sample of purificational procedure was used at 3 days of germinated corn.

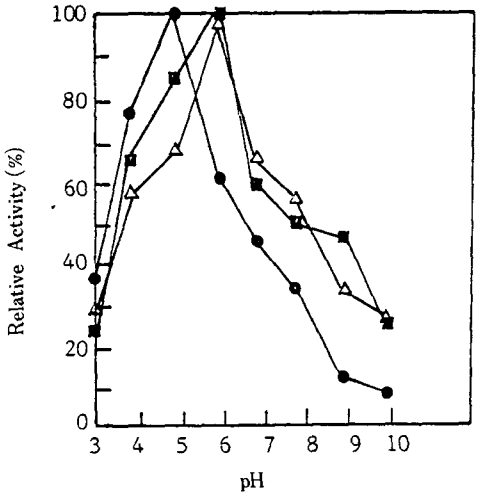

Fig. 1. Effect of pH on the activity of amylase I, II and III
—△— : Amylase I,
—●— : Amylase II,
—■— : Amylase III

Enzymatic properties

1) Effects of pH and temperature on the activities of the amylase(I), (II) and (III)

The amylases activities were measured at various pHs from 3 to 10, respectively. The optimum pH for the activity was found to be around pH 6, pH 5 and pH 6(Fig. 1).

The enzyme activity was measured at various temperatures from 25℃ to 85℃. The optimum temperature for activity was around 35℃, 55℃ and 55℃, respectively(Fig. 2).

2) Effects of pH and temperature on the stability of the amylase(I), (II) and (III)

The enzyme solutions were maintained at various pHs, ranging from pH 3 to pH 10, respecti-

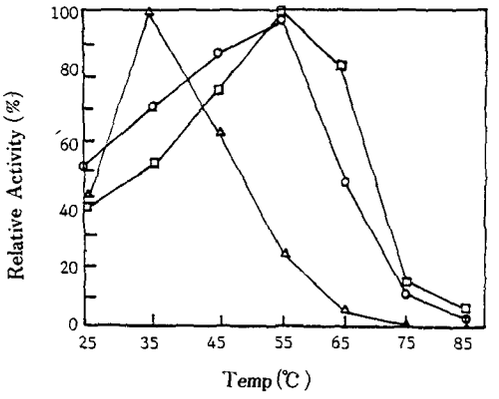

Fig. 2. Effect of temperature on the activity of amylase I, II and III
-△- : Amylase I,
-○- : Amylase II,
-□- : Amylase III

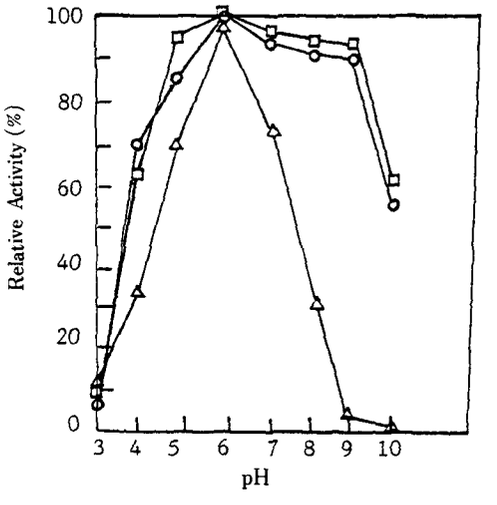

Fig. 3. Effect of pH on the stability of amylase I, II and III
-△- : Amylase I,
-○- : Amylase II,
-□- : Amylase III

vely for 1hr at 35℃ and then the remaining activity was assayed. The enzyme was stable between pH 5 to pH 8, pH 4 to pH 10 and pH 4 to pH 10(Fig. 3). the enzyme was treated in a water bath at various temperatures from 25℃ to 85℃ for 30min at pH 6 and then the enzymes were completely inactivated by the same treatment at 75℃(Fig. 4)

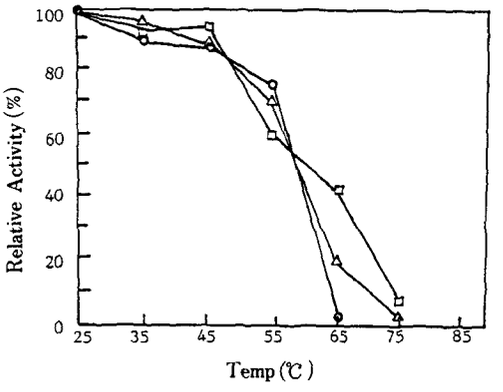

Fig. 4. Effect of temperature on the stability of amylase I, II and III
-△- : Amylase I,
-○- : Amylase II,
-□- : Amylase III

3) Effects of alcohols and $CaCl_2$ on the stability of amylase(I), (II) and (III) in the course of storage time

As shown(Fig. 5) the purified amylases were stable with $CaCl_2$. therefore, the phosphate buffer contained 1mM $CaCl_2$ for the purification procedure.

4) Action mode of purified amylase(I), (II) and (III)

Identification of sugars formed by the purified

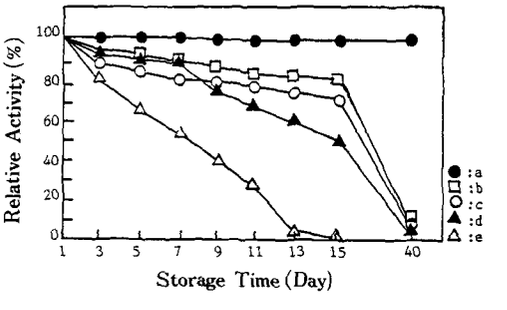

Fig. 5. Effects of alcohols and $CaCl_2$ on the stability of the amylase in germinating corn
a : $CaCl_2$(0.001M)
b : Ethylen glycol(5%)
c : Glycerin(5%), d : Ethanol(5%)
e : None

amylases reactions. Purified amylases hydrolyzed 5%-soluble starch to monosaccharides and oligosaccharides. Thus, the reaction was then carried out at 35℃ for 4hr on the water bath. The reaction mixture was heated in a boiling water for 10min to inactive the enzymes and then centrifuged to remove very small amounts of insoluble materials from the hydrolysate. A small quantity of the supernatant was analyzed by HPLC(Waters, Co., Ltd. : bio Rad HPX-87 column, RI detector, elution was performed with the solvent of distilled water at the flow rate of 0.7mℓ/min, 120kg/cm pressure). As shown Fig. 6, hydrolysis of 5%-soluble starch was carried out and percentage was determined by the method of blue value.

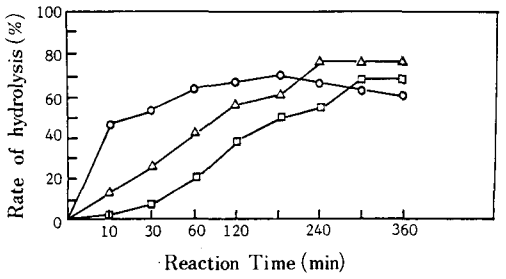

Fig. 6. Hydrolysis of soluble starch by the amylase I, II and III in germinating corn. Hydrolysis of soluble starch(5%) was carried out at 35℃, pH 6.0 using amylase I, II and III. Hydrolysis percentage was determined by the method of blue value.
-△- : Amylase I,
-○- : Amylase II,
-□- : Amylase III

As shown Fig. 7, sugars hydrolyzed by the amylases action and amylase(I) reaction led to the appearance of DP_4, DP_3, maltose and glucose.

Therefore, the pufified amylase(I) was identified as a kind of α-amylase. But amylase(II) and (III) were enzymes producing maltotetrose as main product.

Table 2. Effect of metal salts on the amylase I, II and III relative activity

Metal salts	Amylase I		Amylase II		Amylase III	
	1mM	0.1mM	1mM	0.1mM	1mM	0.1mM
None	100	100	100	100	100	100
$AgNO_3$	78	88	96	92	76	78
$BaCl_2$	103	105	103	98	105	99
$CaCl_2$	120	116	129	124	135	120
$CoCl_2$	119	110	120	119	82	87
$CuCl_2$	120	113	70	40	78	51
$K_2Cr_2O_7$	110	101	101	120	92	89
$FeCl_3$	118	103	131	130	110	124
$HgCl_2$	40	32	25	50	7	57
$MgSO_4$	38	30	60	51	25	35
$MgCl_2$	90	88	95	103	80	90
$MnCl_2$	28	20	26	25	31	22
$NaNO_3$	60	55	96	101	64	53
NaCl	110	102	98	100	100	102
$SnCl_2$	29	40	23	43	57	61
$ZnCl_2$	36	31	38	26	30	20
$NiCl_2$	78	70	92	113	40	43
EDTA	30	40	70	85	60	55

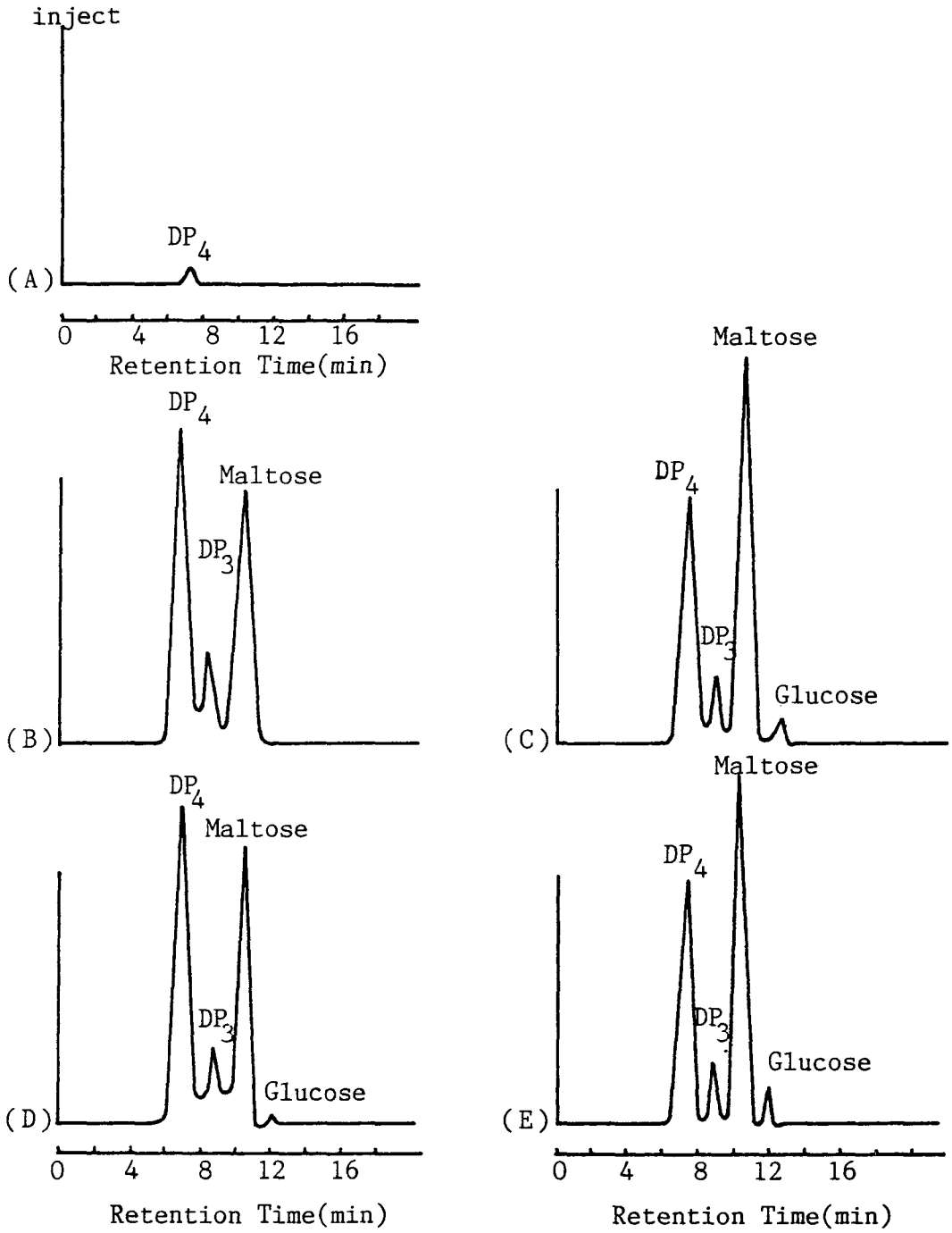

Fig. 7. Hydrolysis of soluble starch by amylase I in germinating corn
(A) : blank
(B) : reaction for 30 min.
(C) : reaction for 60 min.
(D) : reaction for 120 min.
(E) : reaction for 180 min.

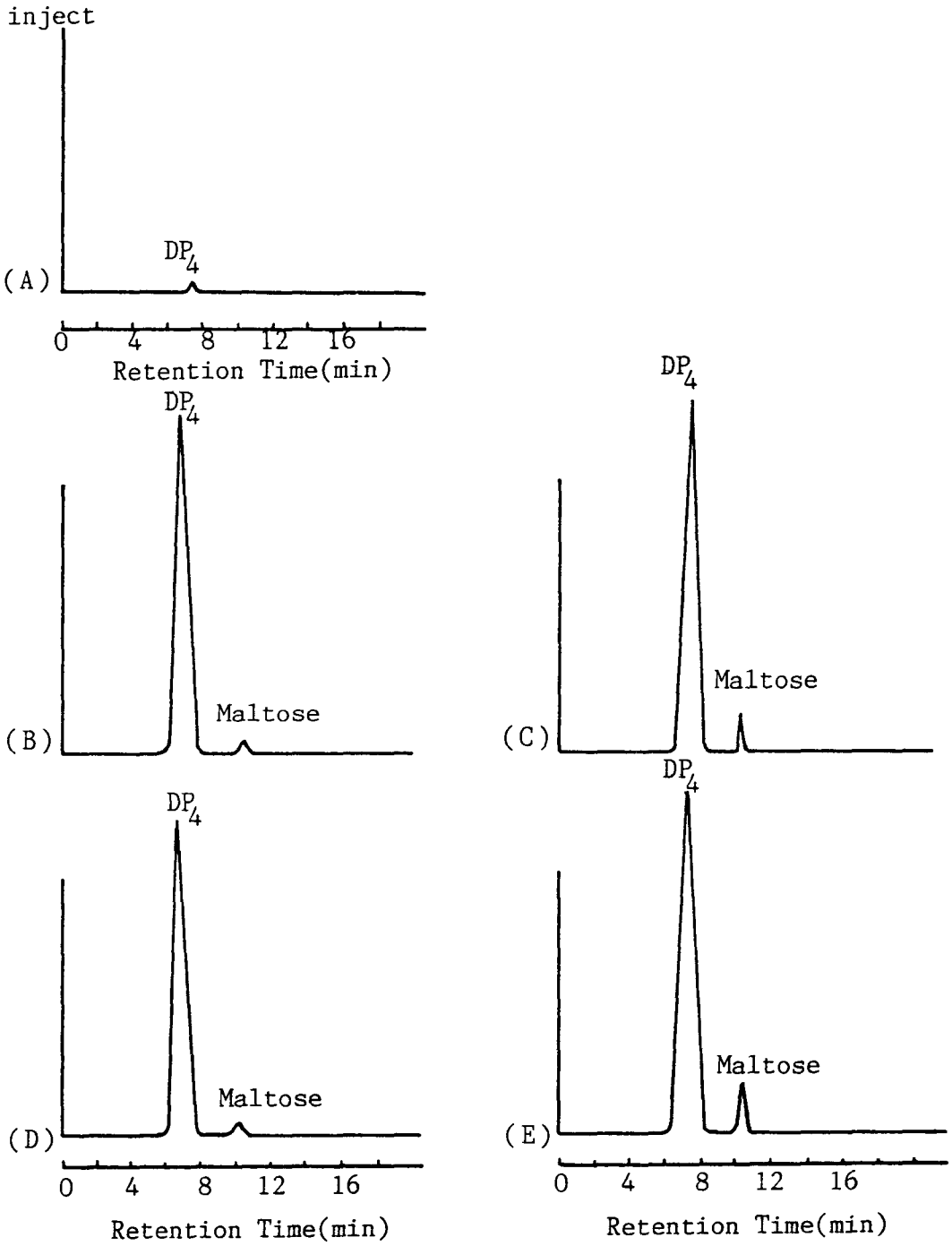

Fig. 8. Hydrolysis of soluble starch by amylase II in germinating corn
(A) : blank
(B) : reaction for 10 min.
(C) : reaction for 30 min.
(D) : reaction for 120 min.
(E) : reaction for 240 min.

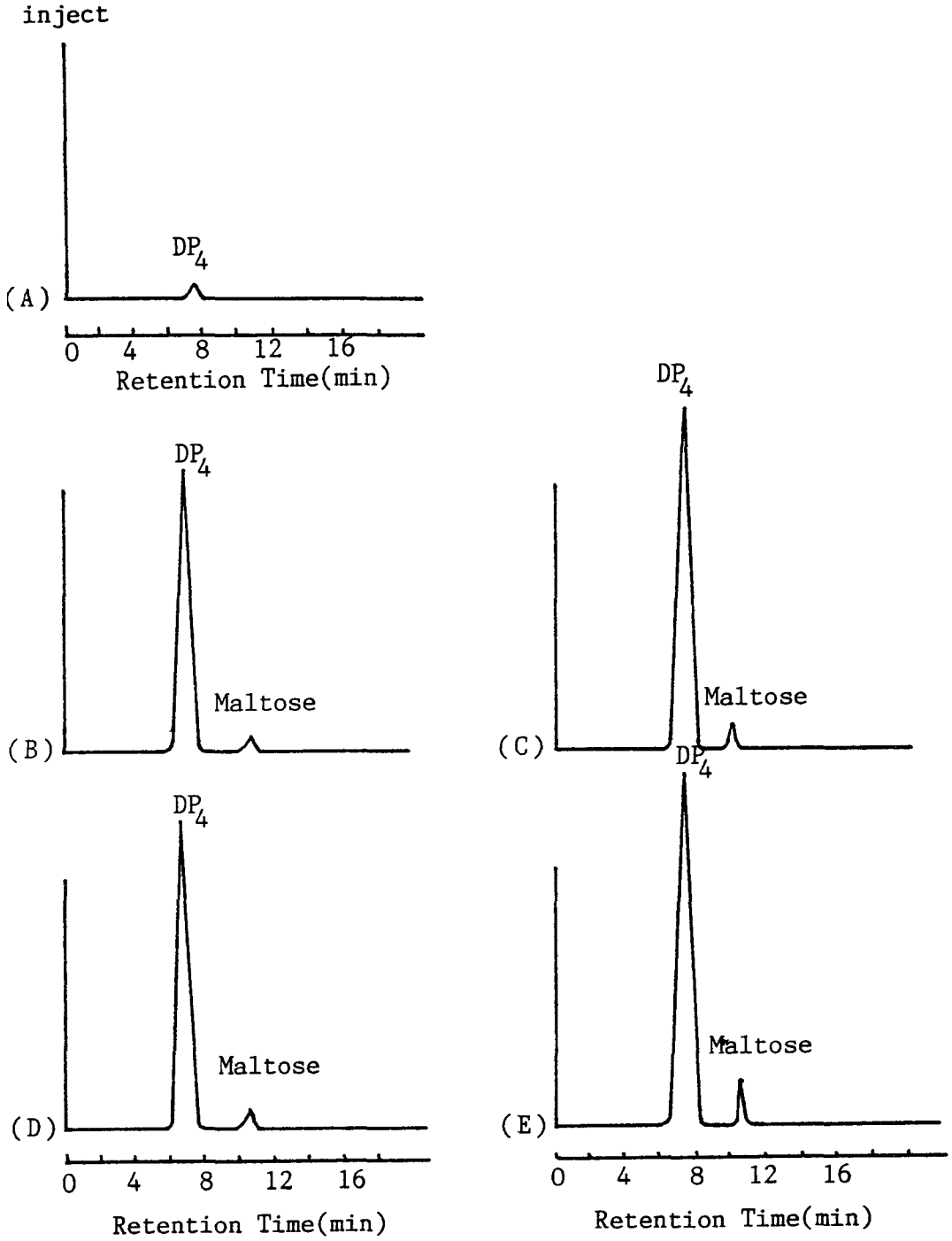

Fig. 9. Hydrolysis of soluble starch by amylase III in germinating corn
(A) : blank
(B) : reaction for 10 min.
(C) : reaction for 30 min.
(D) : reaction for 120 min.
(E) : reaction for 240 min.

But amylase(II) was different from amylase (III) by some biological properties. On the other hand, as shown(Table 2), the amylase(I), (II) and (III) activity increase in presence of 1mM and 0.1mM of Ba, Ca, Co and Fe groups, but was inhibited in that of Ag, Sn, Hg and Zn groups.

The most of amylases may possess common properties of M. W, pH, temperature and inhibitor[21]. It was all much the same such as sweet potato(optimal pH ; pH 4-5, M. W ; 52,000), wheat(pH 5.2 ; 64,200), barley(pH 6 ; 61,000). We described all these obsrvations such as some features of the partially purified amylases in germinating corn seeds in this paper.

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발아 옥수수 amylases의 정제 및 특성

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요 약

발아중인 옥수수에서의 전분의 가수분해효소인 amylases의 종류를 규명하고자 황산 암모늄염석법, DEAE-Sephadex A-50을 이용한 이온교환칼럼법과 Sephadex G-100 Gel filtration chromatography방법으로 정제하였으며 전분가수분해효소는 3개의 peak가 나타났으며, 이를 각각 모아서 정제한 결과 각각의 비활성은 70.47(units/㎎), 62.98(units/㎎), 80.39(units/㎎)으로, 저단백식품의 하나인 옥수수임에도 불구하고 높은활성을 나타내었다. 이것은 발아중 가수분해되는 전분체내에 이 효소의 작용이 커졌음을 유리당의 양이 증가하였음으로 알 수 있었다. 또한 고속액체크로마토그라피를 이용하여 분석한 결과 3종류의 amylases중에 amylases(Ⅰ)은 α-amytotetrose의 종류로 밝혀졌으며, amylase(Ⅱ)와 (Ⅲ)는 각각, 주로 maltotetrose의 단위로 가수분해하는 전분 분해 효소이나, 서로 생물학적 성격에서 약간씩의 차이를 보이므로 같은 종류는 아닐 것으로 사료되었다.