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Hydrolytic Patterns of 11S Globulin (Glycinin) by Soymilk-Clotting Enzymes I and II

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Abstract

Hydrolytic patterns of 11S globulin (glycinin), storage protein of soybean, by soymilk-clotting enzymes I and II from *Bacillus* sp. K-295G-7, which was the first soymilk-clotting enzyme to be found in a bacteria, was investigated. The clotting time of about 4~5 min is revealed by the Enzymes I and II(0.025 units at 35° C) on the acidic subunit. In electrophoresis, acidic subunit (A_3, M.W. 45,000) disappeared almost completely within 2 min and new products corresponding to the molecular weight of 16,000 and 20,000 were formed by the action of Enzymes I and II. Furthermore, Enzyme II produced a degradation compound having a molecular weight of about 30,000. In contrast, the hydrolytic patterns of basic subunit (M.W. 20,000) by Enzymes I and II were similar, but Enzyme II produced low molecular weight products slower than that of Enzyme I.

Key words : glycinin, acidic subunit, basic subunit, soymilk-clotting enzyme

INTRODUCTION

Soybean has a few shortcomings, such as beany flavor, lower protein recovery, water absorption and retention in the gel used for food stuffs. In order to improve these weakpoints of soy protein, many workers[1~4] have attempted to investigate the physical, chemical and enzymatic modifications. It has been reported that bromelain, a plant proteinase, is capable of clotting the protein in soymilk, thereby forming soymilk curd. Fuke et al.[4] have investigated the production of cheese-like food from soymilk by treament of bromelain. However, the cheese-like product with bromelain has a problem of being slightly bitter. The author reported the screening of microorganisms producing soymilk-clotting enzyme from soil, and the production and investigation of some properties of the enzyme. Also the curd made with the enzyme from *Bacillus* sp. K-295G-7 has a smooth texture and a mild taste without any bitterness was reported[5]. The author has undertaken the purification of the enzyme having high soymilk-clotting activity[6], and clarified the substrate specificity of Enzymes I and II on insulin B chain and other peptide hormones and compared with those of other proteinases having soymilk-clotting activity[7].

The mature soybean seed contains two storage proteins, glycinin and conglycinin, which have sedimentation coefficients of 11S and 7S, respectively. Glycinin, the most abundant storage protein of soybean, has molecular weight of approximately 350,000 and consists of 12 subunits[8,9] i.e. six acidic subunits (M.W. 37,000~45,000) and six basic subunits (M.W. 20,000~22,000). It was demonstrated that each acidic subunit is linked to the specific basic subunit via disulfide bridges[10]. However, the precise number of acidic and basic subunits remains in controversy, and may well vary between cultivars[11,12].

Although partial and complete amino acid sequence determinations of each subunit of glycinin were reported[13,14], degradation patterns of these subunits of glycinin by soymilk-clotting enzyme remain to be established. In order to, therefore, clarify the disintegration process of acidic and basic subunits by soymilk-clotting enzyme, the author intended to investigate the degradation of acidic and basic subunits with soymilk-clotting Enzymes I and II by elctrophoretic changes.

MATERIALS AND METHODS

Preparation of enzyme

Three liters of enzyme production medium [yeast extract (0.2%)-peptone (0.2%)-glucose (0.5%)-potassium phosphate (0.5%)-agar (1.5%)], containing a small amount of Toshiba silicone defoamer, was poured into a 5-liter jar fermentor, and sterilized at 120° C for 15min in an autoclave. The seed culture(200ml) of *Bacillus* sp. K-295G-7, which had been grown in the same medium in 500ml shaking flasks at 35° C for 2days on a reciprocal shaker, was inoculated into the fermentor. The cultivation was carried out at 35° C with an aeration rate of 1.5 liters/min and with agitation at 650rpm.

Purification of soymilk-clotting enzyme

Enzymes I and II from *Bacillus* sp. K-295G-7 (the isolated microorganism belonged to the gram-positive *Bacillus* species and it produced alkaline proteinase was found) was purified by the methods described previously[6]. During the purification steps of the enzyme, the soymilk-clotting activity was separated into two fractions which were designated as Enzyme I and Enzyme II in the order of elution.

Preparation of glycinin

Glycinin (11S) and its acidic and basic subunits were isolated by using the method of Moreira et al.[13]. The defatted flour (*Glycine max.* Tamahomare) was treated with 0.035M potassium phosphate buffer solution (pH 7.6) containing 10mM 2-mercaptoethanol and 0.4M sodium chloride (buffer A). the resultant crude 11S globulin solution was applied to the Sepharose 6B column (2.5 × 100cm) and 11S globulin fraction was obtained. This preparation contained 95% glycinin (11S) as determined by gel electrophoresis.

Reduction and alkylation of glycinin

The glycinin obtained above was reduced by the procedure described by Kitamura et al.[8] and Kim and Kinsella[15]. Soybean glycinin was reduced with DTT, and the SH-groups thus formed were blocked with iodoacetamide to prevent disulfide interchange reaction.

Preparation of the acidic subunits from alkylated glycinin using DEAE-Sephadex chromatography

Three hundred milligrams of alkylated glycinin were applied to a column (2.5 × 40cm) previously equilibrated with 0.035M potassium phosphate buffer solution containing 6M urea and 20mM 2-mercaptoethanol (pH 6.6). Fraction F_0 was passed through the column, and then fractions (F_1 to F_3) were eluted from the column using a 1-liter linear gradient from 0 to 0.3M sodium chloride in the same buffer solution.

Preparation of the basic subunits from alkylated glycinin using CM-Sephadex chromatography

One hundred miligrams of a mixture of basic subunits from the DEAE-Sephadex chromatography (Fo) were applied to a CM-Sephadex column (2.5 × 40cm) previously equilibrated with 0.035M potassium phosphate buffer solution(pH 6.6) containing 6M urea and 20mM 2-mercaptoethanol. A 1-liter linear gradient from 0 to 0.2M sodium chloride in the same buffer solution eluted the basic subunits from the column.

Soymilk-clotting activity

Soymilk-clotting activity was determined according to the method described previously[5].

Action of soymilk-clotting enzymes on acidic subunit (A_3)

A solution of acidic subunit (2ml, 0.55% w/v) in 0.04M potassium phosphate buffer solution (pH 6.1) was mixed with 0.2ml of enzyme solution (0.025unit) and incubated at 35° C. The reaction mixture (0.2ml) was taken at various times, and the reaction was stopped by mixing with 20μl of 5% DFP, a serine protease inhibitor. Then the samples were analyzed by SDS-PAGE.

Gel electrophoresis

SDS-PAGE electrophoresis is performed in 12.5% and 14.5% slab gel by the method of Laemmli[16].

N-Terminal amino acid analysis

The N-terminal amino acid of each polypeptide was determined by the method of Hartley[17].

RESULTS AND DISCUSSION

Preparation of acidic and basic subunits of glycinin

SDS-PAGE analysis of the partial purified glycinin showed major bands at MW=42,000 (C and D in Fig. 1), 45,000 (E in Fig. 1), and 20,000 (F and G in Fig. 1). Therefore, the glycinin was used following experiments, because no conspicuous band was observed at MW=54,000, which corresponded to α and α' subunits of conglycinin. The fractionation profile of the acidic subunits on DEAE-Sephadex A-50 column chromatography gave three peaks (Fig. 2). The first peak, F_o, was passed through the column and corresponded to a mixture of the basic subunits (M.W. 20,000). The separation of the other fractions, F_1 and F_2, required linear increasing of sodium chloride concentration (Fig. 2). However, these fractions contained two components of each other (data not shown). Acidic subunits, F_1 and F_2, were estimated to be molecular weight of 42,000 on SDS-PAGE, while acidic subunit F_3 had a molecular weight of about 45,000 (E in Fig. 1).

As shown in Fig. 3, the basic subunits were purified on CM-Sephadex C-50 column chromatography and separated into two peaks (F_4 and F_5 in Fig. 3). Basic subunit, F_5, appeared to be slightly smaller than the other basic subunits (G in Fig. 1).

N-Terminal amino acid determination

N-Terminal amino acids of isolated acidic subunit and basic subunit coincided well with the report[13]. *N-terminus of F_3 was isoleucine, which coincided* with that of A_3. While when analyzed on SDS-PAGE, basic subunits could be identified based on mobility. Basic subunit, F_5, contained glycine as N-Terminus ; it was almost similar to B_3.

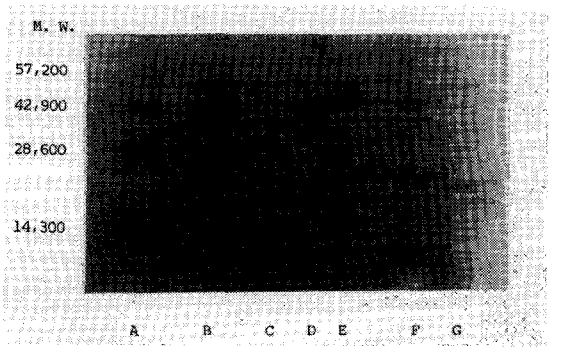

Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified soybean glycinin (11S).
A, molecular weight markers ; B, purified 11S ; C, acidic subunits F_1 ; D, F_2 ; E, F_3 ; F, basic subunits F_4 ; G, F_5.

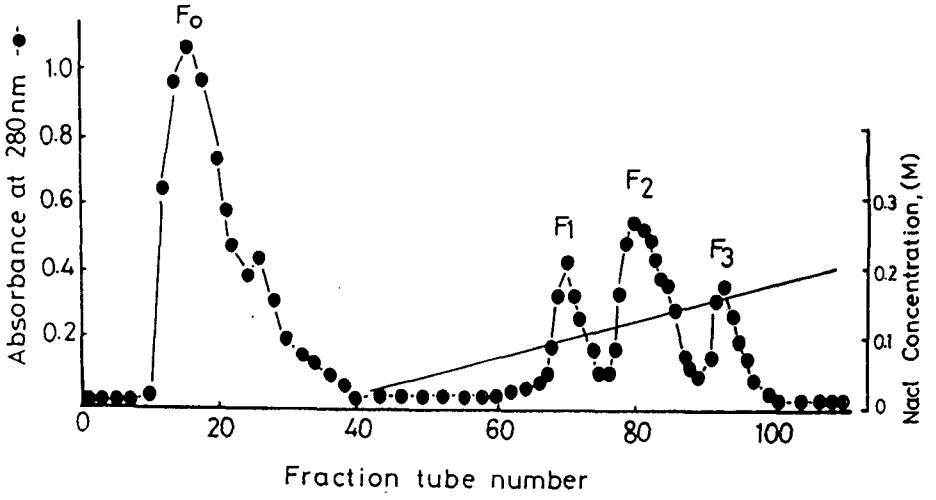

Fig. 2. The elution profile of the acidic subunits on DEAE-sephadex column chromatography.
Degradation products were eluted by 500ml each of a NaCl concentration gradient (0~0.3M). The flow rate was 12.5ml/hr and 6.5ml fractions were collected.

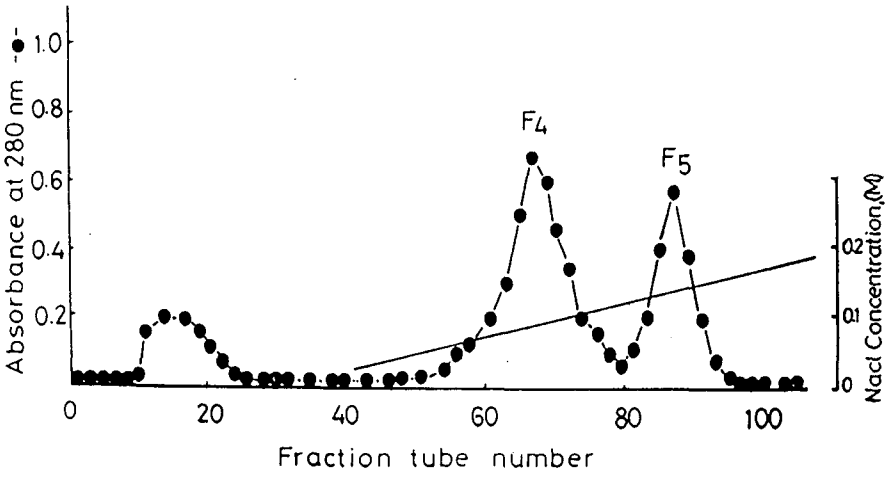

Fig. 3. The elution profile of the basic subunits on CM-Sephadex column chromatography.
Degradation products were eluted by 500ml each of NaCl concentration gradient (0~0.2M). The flow rate was 12.5ml/hr and 6.5ml fractions were collected.

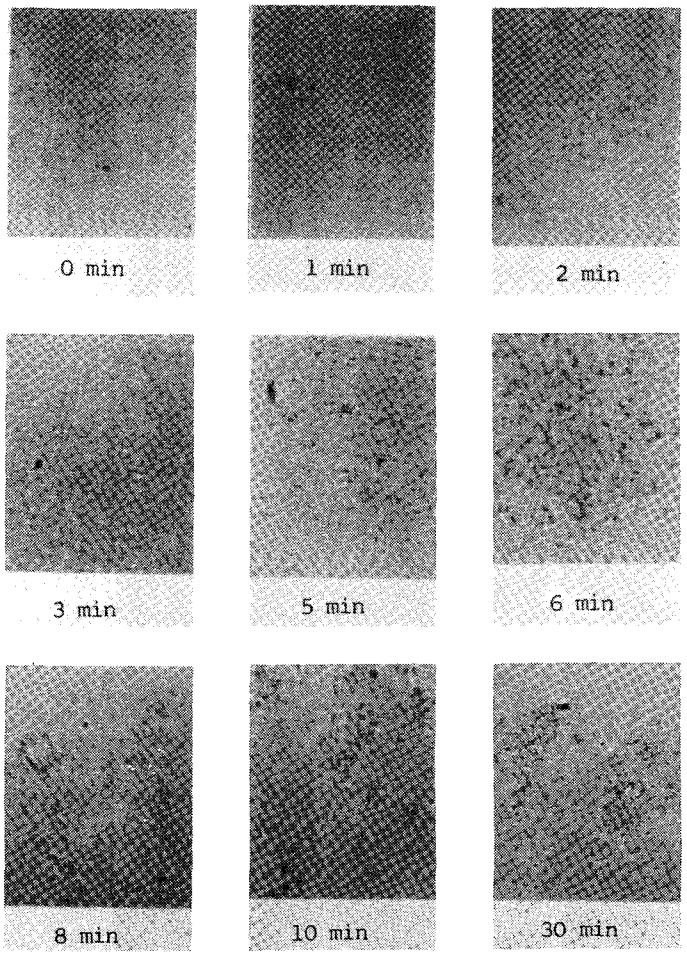

Fig. 4. Coagulum formation of acidic subunit (A_3) by enzyme I (magnification, ×40).

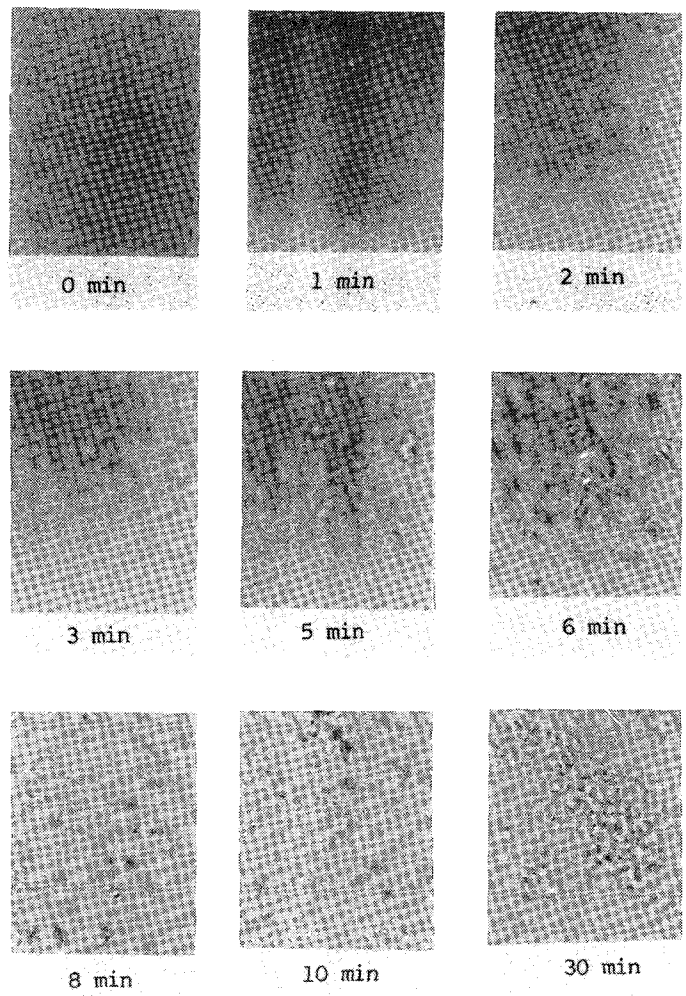

Fig. 5. Coagulum formation of acidic subunit (A_3) by enzyme II (magnification, $\times 100$).

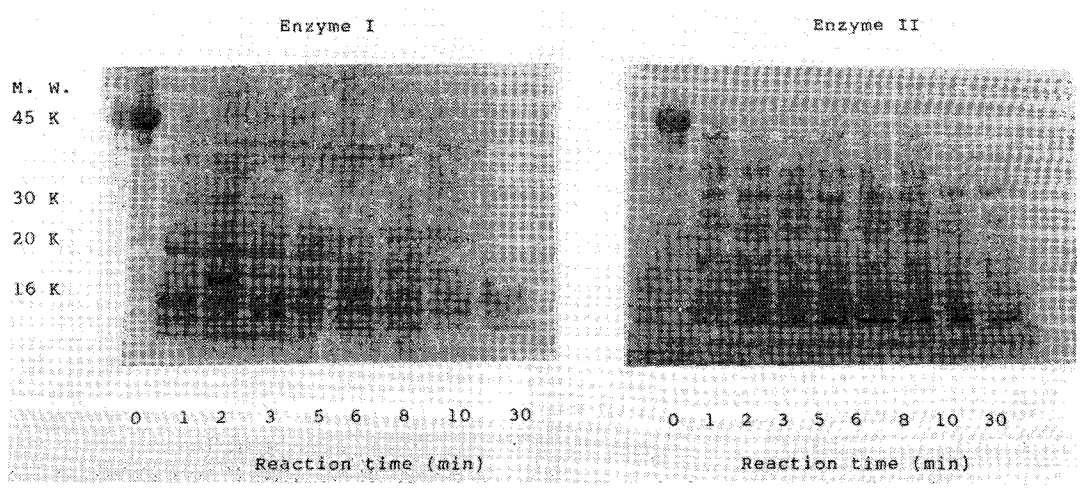

Fig. 6. The degradation patterns of acidic subunit (A_3) by enzyme I and II.

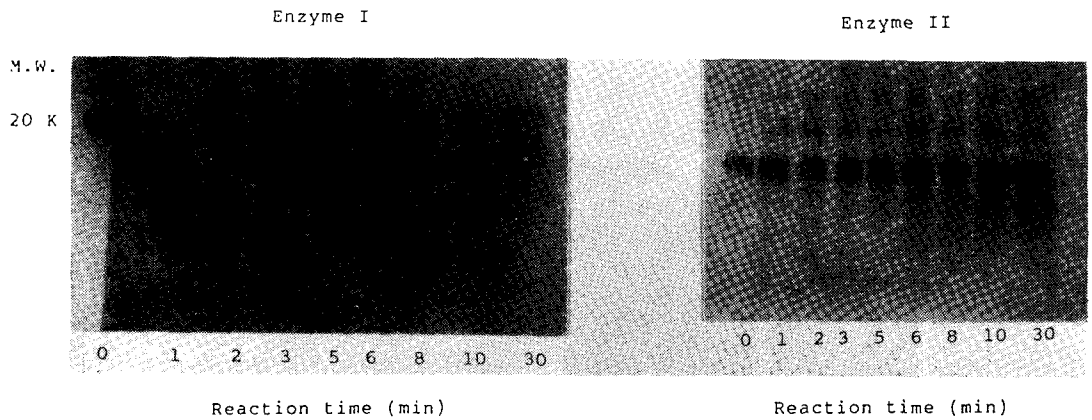

Fig. 7. The degradation patterns of basic subunit (B_3) by enzyme I and II.

Action of soymilk-clotting enzymes on acidic subunit (A_3)

As shown in Figs. 4 and 5, 0.025 units of Enzymes I and II were found to give a clotting time of 4~5min. In SDS-PAGE (Fig. 6), acidic subunit A_3 (M.W. 45,000) almost completely disappeared within 2 min and new bands corresponding to 16,000, 20,000 were formed by the actions of Enzymes I and II. In addition, Enzyme II yielded a degradation product having a molecular weight of 30,000 and this was observed not only at the early stage of proteolysis but also after a long time of incubation. During the entire course of the reaction, the results indicate that the degradation patterns of Enzymes I and II differed from each other. Meanwhile, the proteolytic patterns of basic subunit by Enzymes I and II were similar, but Enzyme I produced low molecular weight products faster than that of Enzyme II (Fig. 7). Considering the above properties, Enzyme II was found to be superior than Enzyme I in terms of substrate specificity.

Mohri and Matsushita[3] reported that since the basic subunits are surrounded by acidic subunits in native 11S globulin, they are not attacked by bromelain easily. However, when the 11S globulin was denatured by heat treatment, basic subunits may be attacked facilely by bromelain as they contain more basic amino acids than acidic subunits. Contrary, Figs. 6 and 7 shows that Enzymes I and II prefered acidic sububits to basic subunits of 11S globulin. This fact indicates that the mechanism of soymilk-coagulation by Enzymes I and II was different from that of bromelain.

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두유응고효소 I 및 II에 의한 11S 단백질(Glycinin)의 가수분해 패턴

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요 약

박테리아 *Bacillus* sp. K-295G-7이 생산하는 두유응고효소 I 및 II 에 의한 11S globulin (glycinin)의 가수분해 패턴을 조사하였다. 효소 I 과 II에 의한 acidic subunit의 응고시간은 약 4~5 분 이었다. 전기영동의 결과, acidic subunit (A_3, MW=45,000)는 효소반응 2분 이내에 완전히 가수분해 되어 분자량 16,000, 20,000의 새로운 band를 형성하였다. 한편 효소 II의 작용으로 약 30,000의 분자량을 가진 분해산물을 생성하였고, 효소 I 과 II의 basic subunit에 대한 가수분해 패턴은 유사하였다.