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Physicochemical Characteristics of Hydrolyzed Soybean Proteins by Immobilized Protease(s)

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고정화 효소를 이용하여 가수분해된 대두단백질의 이화학적 특성

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Abstract

Hydrolysis of soybean proteins was carried out by immobilized trypsin and/or alpha-chymotrypsin. The partially hydrolyzed products of soybean proteins were evaluated for their molecular weights and molecular charges by using Ferguson's plot. The ratio of average molecular weights to average molecular charges($\overline{M}/\log \overline{Y}_o$) of modified soybean proteins could be used to predict functional properties such as solubility, water holding capacity, oil holding capacity, and emulsifying ability. The low ratio of modified soybean proteins indicated high solubility, while the high ratio showed high water holding capacity. The appropriate ranges of the ratios were necessary for maximun oil holding capacity and emulsifying ability.

Key words : average molecular weight, average molecular charge, percent T, percent C

Introduction

The modification of plant proteins has received much attention for their direct consumption. However, desirable functional properties of soybean proteins should be adopted to achieve the best results as food ingredients.

Enzymatic modification has some advantages[1]. Moreover, the use of immobilized proteases can solve the inherent problems associated with using free enzyme[2].

Electrophoresis can be carried out for evaluating proteins either synergistically or independently of charge and size[3]. When logarithm of relative mobility for a single protein was plotted opposed gel concentration, a linear relationship was found as first described by Ferguson on starch gel[4]. Later, this relationship was independently found on polyacrylamide gel[5]. The retardation coefficient(slope) is related to molecular size and the relative free mobility(y-intercept) is related to molecular net charge[6]. Anderson[7] examined water-extractable, acid-precipitable and whey proteins from soybeans by disc gel electrophoresis in pH 8.9 tris-glycine buffer, at gel concentrations ranging from 4 to 13%.

The objectives of this study were to understand the molecular properties of soybean proteins modified by immobilized protease(s) and to predict the potential applications of modified proteins.

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Materials and Methods

Immobilization of enzymes

The nylon pellets(2.8± 0.4 mm L, 2.0 mm OD) were used as supports for trypsin(Wortington Biochem. Corp.) and alpha-chymotrypsin(Pfaltz and Bauer Inc.). Enzymes were bound through the Schiff's base to nylon pellets by a modification of the techniques described by Smiley *et al*[8]. Nylon pellets were perfused at 45℃ for 1 hr with a methanolic solution containing $CaCl_2$ to remove amorphous nylon. After rinsing them, the pellets were treated with 3.65 N HCl at 50℃ for 2 hr. The pellets were throughly washed with water until free of Cl^-, and then treated overnight with 8% glutaraldehyde in 0.5 M sodium phosphate buffer(pH 7) at 25℃. Excess glutaraldehyde was rinsed out with water and then, a solution containing 2 mg/ml of enz-

yme in 0.1 M phosphate buffer(pH 7) was treated at 5℃ for 40 hr. Alternate washing with water and 0.1 M NaCl removed excess enzyme. Final washing was done by recycling the substrate.

Soybean protein isolate

Soybean seeds were cracked, dehulled, ground and defatted with hexane according to the method of Wolf[9]. Defatted soybean meal was treated with 5 parts of water(pH 8) adjusted by 0.1 N NaOH for 1 hr and centrifuged at 8,000×g for 20 min. The extract was adjusted to pH 4.5 by 0.1 N HCl and centrifuged at 8.000×g for 20 min. The precipitate was suspended in water and adjusted to pH 7. The suspension was boiled for 40 min to inactivate protease inhibitors, and then freeze-dried.

Batch stirred tank reactor

The soybean protein was dispersed in 0.07 M Sorenson's phosphate buffer(pH 7.5, 2.5% suspension, w/w). A 250 ml Erlenmeyer flask containing substrate and immobilized enzyme(s) was placed in a water bath shaker. The agitator was rotated at 200 rpm for various incubation time at 55℃. The control sample was prepared without adding enzyme.

Determination of amino groups

The new amino groups formed by hydrolysis were determined by using the 2,4,6-trinitrobenzenesulfonic acid reaction[10].

Evaluation of molecular sizes and charges

Polyacrylamide disc-gel electrophoresis(PAGE) was carried out in principle as described by the Davis method[11] and the modified Davis method[12]. The Davis method involved an electrophoresis system consisting of various gel concentrations ranging from 5.25% to 16.00% T in separating gel(pH 8.9), 2.5% C in stacking gel(pH 6.7), and tris-glycine electrode buffer, pH 8.3, using the terminology introduced by Hjerten[13]. Gel concentration(%T) and crosslinkage(%C) are given as :

$$\%T = (\text{g acrylamide} + \text{g N,N'-methylenebisacrylamide, BIS}) \times 100/100\ \text{ml solution}$$

$$\%C = (\text{g BIS}) \times 100/(\text{g acrylamide} + \text{g BIS})$$

The proteins(1~2 mg/ml) were dissolved in 0.062 M tris-buffer(pH 6.7), 1% Triton X−100 and 1% 2-mercaptoethanol.

When log relative mobility for a single protein versus gel concentration is plotted, a linear relationship is found which follows the equation[14] : $\log_{10} R_f = \log_{10} Y_o + K_R T$ where R_f is the relative mobility at gel concentration T, Y_o is the extrapolated relative mobility at zero gel concentration, and K_R is the retardiation coefficient.

The average molecular weight($\overline{M}$) determined by using the gel filtration concept characterized by its principal statistical moments[14] : $\overline{M} = \sum n_i M_i / \sum n_i$ where n_i is the concentration number of molecules of the i-th kind per unit volume and M_i is its molecular weight. Assuming that the standard deviation of the concentration distribution of all stained different species is identical, the concentration of protein or peptide molecules in the i-th interval of a gel is proportional to absorbance(A_i). Therefore, the proportion of the total weight present in the i-th distance(L) is : $n_i = A_i L / \sum A_i L$. Likewise, the proportion of the i-th band is : $n_i = Ar_i / \sum Ar_i$ where Ar_i is area of the i-th band. Therefore, $\overline{M} = \sum A_i M_i / \sum A_i$[15] or $\overline{M} = \sum Ar_i M_i / \sum Ar_i$.

In a similar manner for evaluating the average molecular weight, the average molecular charge can be derived as :

$$\log \overline{Y}_o = \sum Ar_i \log Y_{oi} / \sum Ar_i.$$

Solubility

To 0.1g of modified soybean proteins, 5 ml of 0.07 M Sorenson's phosphate buffer(pH 6.5) were added in a weighted centrifuge tube. After swelling samples for one hour, the solution was agitated with a Vortex mixer(Scientific Industries, Inc.) set at speed 5 for 2 min. The sample was centrifuged at 1,300×g for 10 min and then the amino groups of the supernatant was analyzed.

Water holding capacity

After decanting the supernatant from the solubility determination, the precipitate was weighted and the weight of water bound per gram protein was calculated as water holding capacity[16].

Oil holding capacity

The oil holding capacity was determined in the same manner as the water holding capacity except that corn oil was used, instead of the phosphate buffer[16].

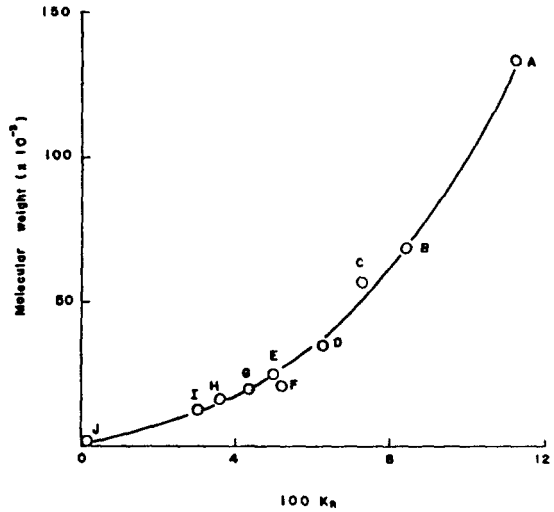

Fig. 1. Relatioonship between K_R and molecular weight for standard proteins

Data of proteins in 1% Triton X-100 and 1% 2-mercaptoethanol.

A=bovine serum albumin(dimer) ; B=bovine serum albumin(monomer) ; C=catalase ; D=pepsin ; E=trypsin ; F=trypsin inhibitor ; G=beta-lactoglobulin ; H=hemoglobin ; I=chymotrypsin ; J=bromophenol blue

Emulsifying ability

The emulsifying ability was estimated by a slightly modified method of Yasumatus *et al*[17]. Two m*l* of corn oil was added to 3 m*l* of 3.3% soybean protein suspension in 0.07 M phosphate buffer(pH 6.5). The mixture was emulsified with a Vortex mixer for 2 min. The emulsion was centrifuged at 1,300×g for 10 min. The emulsifying ability was the ratio of the volume of emulsified layer multiplied by 100 to the whole volume in the tube.

Results and Discussion

In Fig. 1, K_R for marker proteins are plotted against their molecular weights. The retardation coefficients were obtained from the slopes of the Ferguson plot[4]. Since the retardation coefficient and relative modility are independent variables[18], it is possible to have anomalous behavior of a protein in electrophoresis so that K_R has an appropriate relationship to molecular weight, but Y_o differs significantly from standards.

Physical constants of the soybean protein isolate are shown in Table 1. The components with molecular weights of 65,000 and 47,000~55,000 seemed to be

Table 1. Physical constants determined by PAGE for soybean protein isolate

Band	K_R	log Y_o	Molecular weight
1	0.0027	−1.4257	1,000
2	0.0900	−0.2993	79,500
3	0.0806	−0.1585	63,000
4	0.0820	0.0518	65,000
5	0.0697	0.1076	47,500
6	0.0756	0.2165	55,500
7	0.0634	0.2825	40,000
8	0.0696	0.3761	48,000
9	0.0704	0.4327	49,000
10	0.0073	0.0103	2,000

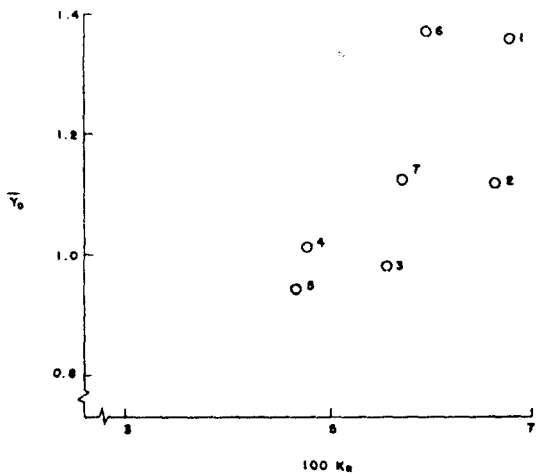

Fig. 2. Reationship between K_R and Y_o estimated from the linear Ferguson plots

Numbers of the modified soybean proteins are : 1=control ; 2=modified by immobilized(IM)-chymotrypsin ; 3=by IM-trypsin-chymotrypsin(1 : 9) ; 4=by IM-trypsin-chymotrypsin(1 : 1) ; 5=by IM-trypsin ; 6 and 7=partially by IM-trypsin-chymotrypsin(1 : 1)

7S and 11S fractions, respectively.

According to Iyengar and Ravestein[19], the disulfide bonds of the glycinin monomer were stable, but the disulfide bridges responsible for polymer formation were reduced at low 2-mercaptoethanol concentration (0.01 M), neutral pH, and ionic strength of more than 0.35, resulting in the decomposition into subunits. The intermediate subunits solubilized in the neutral solvents containing 1% Triton X-100 and 1% 2-mercaptoethanol were presumed to be linked by the electrostatic interactions between acidic and basic subunits.

To determine the relationship between molecular and functional properties, the average retardation coef-

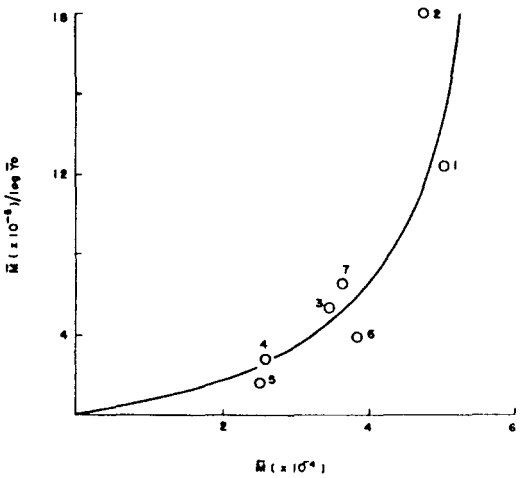

Fig. 3. Relationship between average molecular weight and average molecular charge estimated from the linear Ferguson plots of the modified soybean proteins
Numbers refer to the modified proteins in Fig. 2.

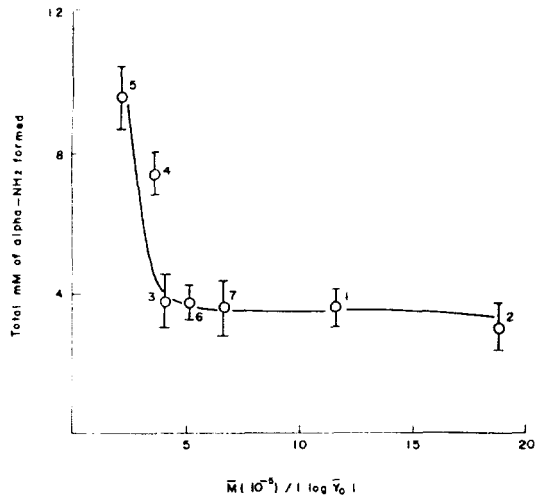

Fig. 4. Solubility of modified soybean proteins as a function of average molecular weight/average molecular charge
Numbers refer to the modified proteins in Fig. 2. The bars indicate standard deviations based on four replications.

ficients and average relative mobilities of the hydrolyzed soybean proteins were calculated by using the weighted average of each component. Fig. 2 shows the relationship between the average molecular charge and the average retardation coefficient for the modified soybean proteins.

When the ratio of the average molecular weights to the average molecular charges of modified soybean proteins are plotted as a function of the average molecular weights, a parabola results as shown in Fig. 3. Hsieh *et al*[15]. reported that there was a linear inverse relationship between molecular weights and solubility. Moreover, proteins with high molecular charges have high solubilities under specific conditions[2]. This indicates that the average molecular weight to the average molecular charge could be used to describe the functional properties of modified soybean proteins.

The relationship between amino groups of modified soybean proteins and the ratio of the average molecular weight to the average molecular charge is given in Fig. 4. When $\overline{M}/\log \overline{Y}_0$ was above 5×10^5, solubility of soybean proteins, which is determined by measuring amino groups, was not changed. Below a certain point, however, the solubility of the hydrolysate was increased exponentially. This effect may be ascribed to the formation of small fragment of peptides upon hydrolysis. Adler-Nissen and klsen[1] found that the solubility of soybean proteins was generally improved in a wide range of pH upon hydrolyzing the proteins with soluble protease, though the improvement of solubility varied depending on the degree of hydrolysis. The solubility of proteins is a good index to predict the potential application of the proteins because the parameter, solubility, is related to many functional properties such as emulsifying capacity, gelation, foaming capacity, and bitter taste[2]. However, some other functional properties such as water holding capacity, oil holding capacity and foaming capacity do not show any significant relationship with the solubility. In some cases, those functional properties decreased as a proteolysis continued[1,2]. It is, therefore, important to control the mean molecular size of hydrolysate fraction, in order to obtain optimum functional properties for various application.

The relationship between water holding capacity and the ratio of the average molecular weight to the average molecular charge is shown in Fig. 5. The reduction of molecular size and the increase in net molecular charge of soybean proteins reduced their water holding capacity. Therefore, the physical entrapment of water into the protein matrix is an important factor for the water holding capacity. Moreover, a highly soluble protein exhibits poor water binding capacity according to

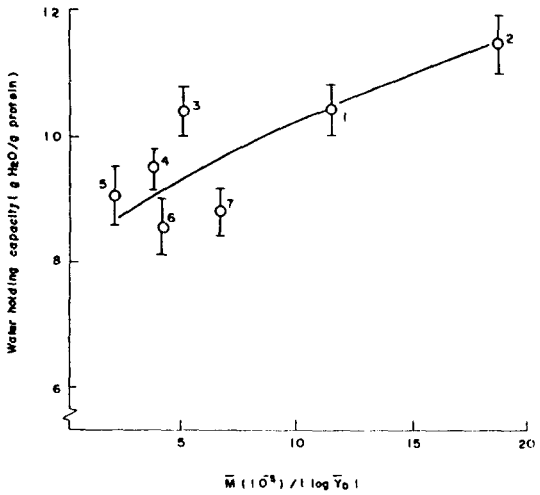

Fig. 5. Water holding capacity of modified soybean proteins as a function of average molecular weight/average molecular charge

Numbers refer to the modified proteins in Fig. 2. The bars indicate standard deviations based on four replications.

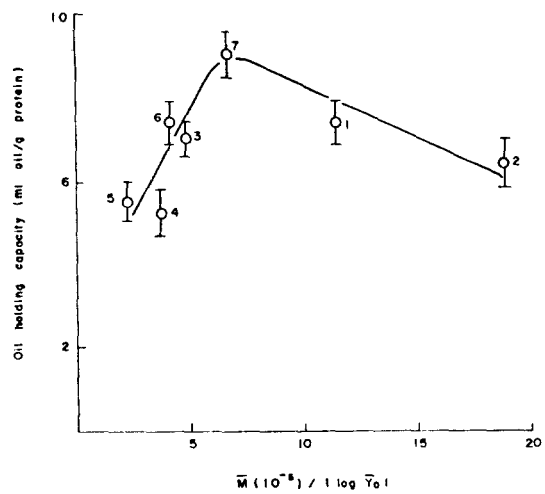

Fig. 6. Oil holding capacity of modified soybean proteins as a function of average molecular weight/average molecular charge

Numbers refer to the modified proteins in Fig. 2. The bars indicate standard deviations based on four replications.

the result of Hermansson *et al*[20]. To achieve maximum water holding capacity of modified soybean proteins, it is necessary to have a large average molecular weight and small net molecular charge.

As demonstrated in Fig. 6, oil holding capacity of modified soybean protein was maximum at 7×10^5 of $\overline{M}/\log \overline{Y}_o$. The excessive decrease in molecular weight and/or increase in the net molecular charge of the protein caused by hydrolysis reduced oil holding capacity. Kabirullah and Wills[21] indicated that the enhanced entrpment of oil was due to the unfolding of the protein structure by succinylation. Thus, the oil holding capacity is affected by both the molecular structure and the macrostructure of proteins.

The emulsifying ability of the modified soybean proteins as a function of the ratio of the average molecular weight to the average molecular charge is shown in Fig. 7. A decrease in emulsifying ability of modified soybean protein was observed where the protein has small molecular size and/or high of low molecular charge. Accordingly, a small molecular size and/or high absolute molecular charge reduced emulsifying ability, and a low net molecular charge decreased emulsifying ability. The simple molecular expansion of soybean protein suspended in water decreased its emulsifying capacity when heated by microwaves or by a conven-

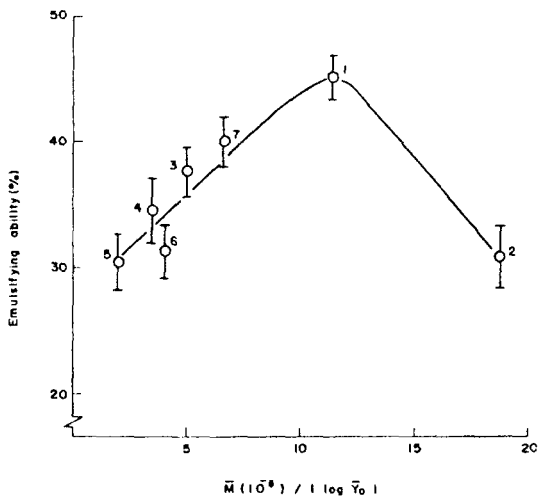

Fig. 7. Emulsifying ability of modified soybean proteins as a function of average molecular weight/average molecular charge

Numbers refer to the modified proteins in Fig. 2. The bars indicate standard deviations based on four replications.

tional method[22]. The emulsifying ability decreased as proteolysis continued, indicating that there is an optimum molecular size and optimum charge of proteins affecting emulsifying ability. Adler-Nissen and Olsen[1] demonstrated that, for optimum emulsification, the hy-

drolysate seemed to have equal amounts of soluble and insoluble material. It is, therefore, important that the molecules not be too small.

요 약

대두단백질의 기능성을 개선하기 위하여 고정화된 트립신 및 키모트립신으로 가수분해시킨 후, 이 대두단백 가수물의 기능성을 조사하였다. Ferguson plot으로부터 가수분해물의 평균분자량 및 평균전하를 계산하여 대두단백의 기능성을 나타내는 인자로 이용하였다. 즉, 단백질의 평균분자량을 평균전하로 나눈 값은 단백가수물의 용해도, 포수력, 포유력, 유화도 등의 기능성을 예측하는데 이용될 수 있었다. 이 분자비가 낮으면 용해도는 향상되었으나 포수력은 감소되었으며 중간범위일 경우 포유력과 유화도는 증가되는 경향을 나타냈다.

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