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Since, glycinin in 6M urea contains only 1.7 SH group/mole, the remaining SH groups are probably the results of the alkaline cleavage of some susceptible disulfide bonds. The implications of the above findings in regard to the mechanism of soybean protein spun fiber formation are discussed.

Additionally, the acidic and basic subunits of glycinin were isolated by a simple ion exchange chromatography method involving a two-step pH change of the elution buffer. Abnormal behavior of the subunits in dodecyl sulfate polyacrylamide gel electrophoresis was suggested.

FINAL REPORT

Contract No. DAHC04 74 C 0029 Good

SULFHYDRYL AND DISULFIDE GROUPS IN GLYCININ; THE MAJOR SOYBEAN PROTEIN

Submitted to

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Summary

The number of disulfide (SS) bonds in glycinin - the major soybean protein - and their rate of cleavage by dithiothreitol (DTT) were determined at various concentrations of urea. A progressive increase in SS bond scission was observed with increasing urea concentration reaching the maximun of 20 SS bonds at 8M urea. In addition, a linear relationship was obtained for the rate of SS cleavage as a function of urea concentration. These results indicate that most of the disulfide bridges are buried in the interior of the protein molecule. Upon exposure to alkaline denaturation, a maximun of 9.2 sulfhydryl (SH) groups were obtained per mole of glycinin at pH 11.9. Since, glycinin in 6M urea contains only 1.7 SH group/mole, the remaining SH groups are probably the results of the alkaline cleavage of some susceptible disulfide bonds. The implications of the above findings in regard to the mechanism of soybean protein spun fiber formation are discussed.

Additionally, the acidic and basic subunits of glycinin were isolated by a simple ion exchange chromatography method involving a two-step pH change of the

elution buffer. Abnormal behavior of the subunits in dodecyl sulfate polyacrylamide gel electrophoresis was suggested. The new method offers a means for obtaining sufficient quantities of these two types of subunits for further biochemical studies.

Introduction

The increased use of soybean proteins in engineered food systems necessitates the study of their structure, properties, and interactions for optimization of their functional characteristics, safe use, and nutritional quality. Additionally, this knowledge can be utilized for modification of their chemical or physical structure in such a manner as to produce new products bearing desirable characteristics as food ingredients. One of the major soybean proteins comprising approximately 70% of all proteins in the seed is glycinin. This protein is expected to be a major constituent of food in the years to come. Therefore, the more we know about this protein at the molecular level, the more effective, economical and safe its utilization will be.

Over the past three years a number of biochemical studies on glycinin in this laboratory were supported totally (Part One) or in part (Part Two) by U.S.A.R.O. Contract No DAHCO4-74-C-0029. These investigations

involved primarily (a) the development of a chromatographic method for the isolation of the acidic and basic subunits of glycinin (b) the measurement of the disulfide bonds and sulfhydryl groups of the native and denatured protein, and (c) the study of its enzymatic hydrolysis by trypsin and pepsin.

PART ONE

SULFHYDRYL AND DISULFIDE GROUPS IN GLYCININ

I. Isolation of the Acidic and Basic Subunits

A. Introduction

Biochemical studies on the structure and properties of the storage proteins of the soybean (*Glycine max*) have important implications in technological and genetic engineering developments in regard to food protein supply. It was originally reported (1,2) that glycinin, the major storage protein of soybean seeds, is composed of acidic and basic subunits. Subsequent studies have indicated that the acidic subunits have higher molecular weight than the basic subunits (3-5) and that some degree of homology exists among the acidic subunits (6). However, there is no general agreement on the number of acidic subunits. Catsimpoolas (1) reported the presence of three acidic and three basic subunits and his findings were later confirmed by Badley *et al*. (5). Kitamura and Shibasaki (4) consider glycinin to be composed of four acidic and three basic subunits. The fourth acidic subunit of 45,000 daltons was previously rejected by Catsimpoolas *et al*. (3) as being part of the structure of glycinin because of its low

concentration in regard to the rest of the acidic subunits. These authors considered the fourth component to be either an association product or a contaminant. Some discrepancy also exists concerning the molecular weight of the subunits. For the acidic subunits, molecular weights of 37,200 (3), 34,800 (5), 37,000 (4) and 45,000 (4) have been found. The basic subunits appear to have molecular weights of 22,300 (3), 22,500 (4) , and 19,600 (5). Amino acid sequence analysis of individual polypeptide chains is still missing.

In view of the importance in understanding the multi-subunitary structure of storage proteins, further studies on the subunits of glycinin are necessary to establish the complete chemical and physical structure of the protein. The first step in such work is the isolation of the individual basic and acidic subunits. This has been accomplished in the past by isoelectric focusing (1) and DEAE-Sephadex A-50 chromatography (4). In attempts to develop a simple procedure for the initial isolation of the two types of subunits (i.e. acidic and basic), we adopted the ion exchange chromatography method of Wright and Boulter (7). This report describes the successful separation of the acidic and basic subunits of glycinin in sufficient amounts to permit

further fractionation within each group by other methods. We also report their molecular weight determination by the use of a discontinuous buffer system in sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

B. Results

Glycinin purified by ammonium sulfate fractionation (8) and subjected to DEAE Bio-Gel chromatography exhibits the typical elution pattern shown in Fig. 1. The barred area indicates the pooled fractions that consist of homogeneous glycinin as determined by electrophoresis. The use of DEAE Bio-Gel instead of DEAE-Sephadex A-50 (9) for the final step in the purification of glycinin has the advantage that regeneration of the Bio-Gel column is more convenient since it does not have to be repacked after each experiment. The disulfide bands of the purified protein were reduced with mercaptoethanol in the presence of 6M guanidine hydrochloride and the liberated sulfhydryl groups were blocked with iodoacetamide. The dissociated protein was subsequently subjected to ion exchange chromatography on a Dowex AG1 (X2) resin in the presence of 6 M urea. Figure 2 shows the separation of two types of subunits. At pH 8.0 the basic subunits which are not retained by

Fig. 1 Purification of glycinin by DEAE Bio-Gel ion exchange chromatography. Column size: 40 x 4 cm. Sample: 400 mg of glycinin in 15 ml of pH 7.6 phosphate buffer containing 0.1 M NaCl and 0.01 M mercaptoethanol. Elution: Same buffer containing NaCl in gradient concentration of 0.1 M to 1.0 M. Flow rate 25 ml/hr. Solid line: uv absorbance at 280 nm. Barred line: pooled fractions containing pure glycinin.

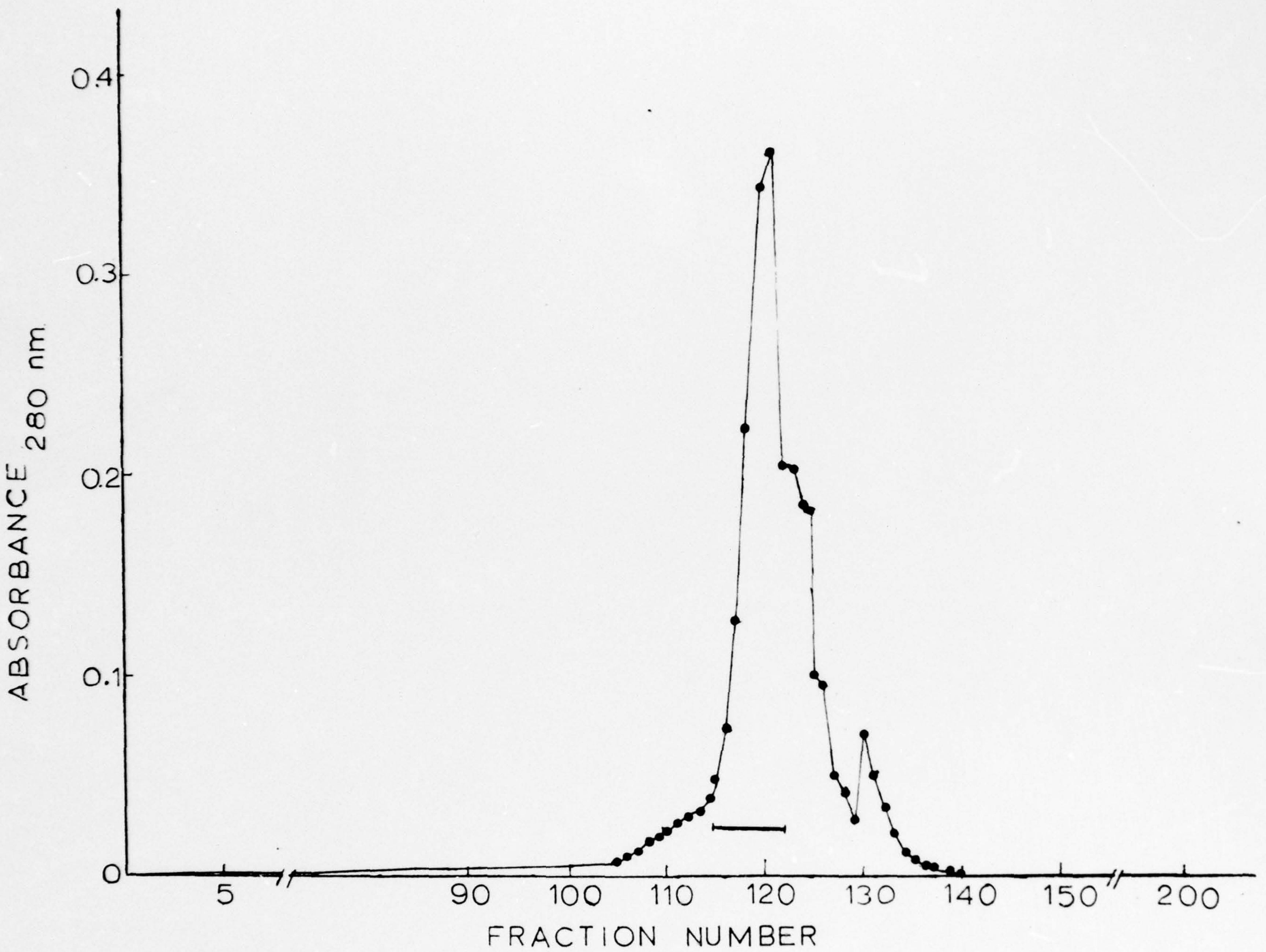
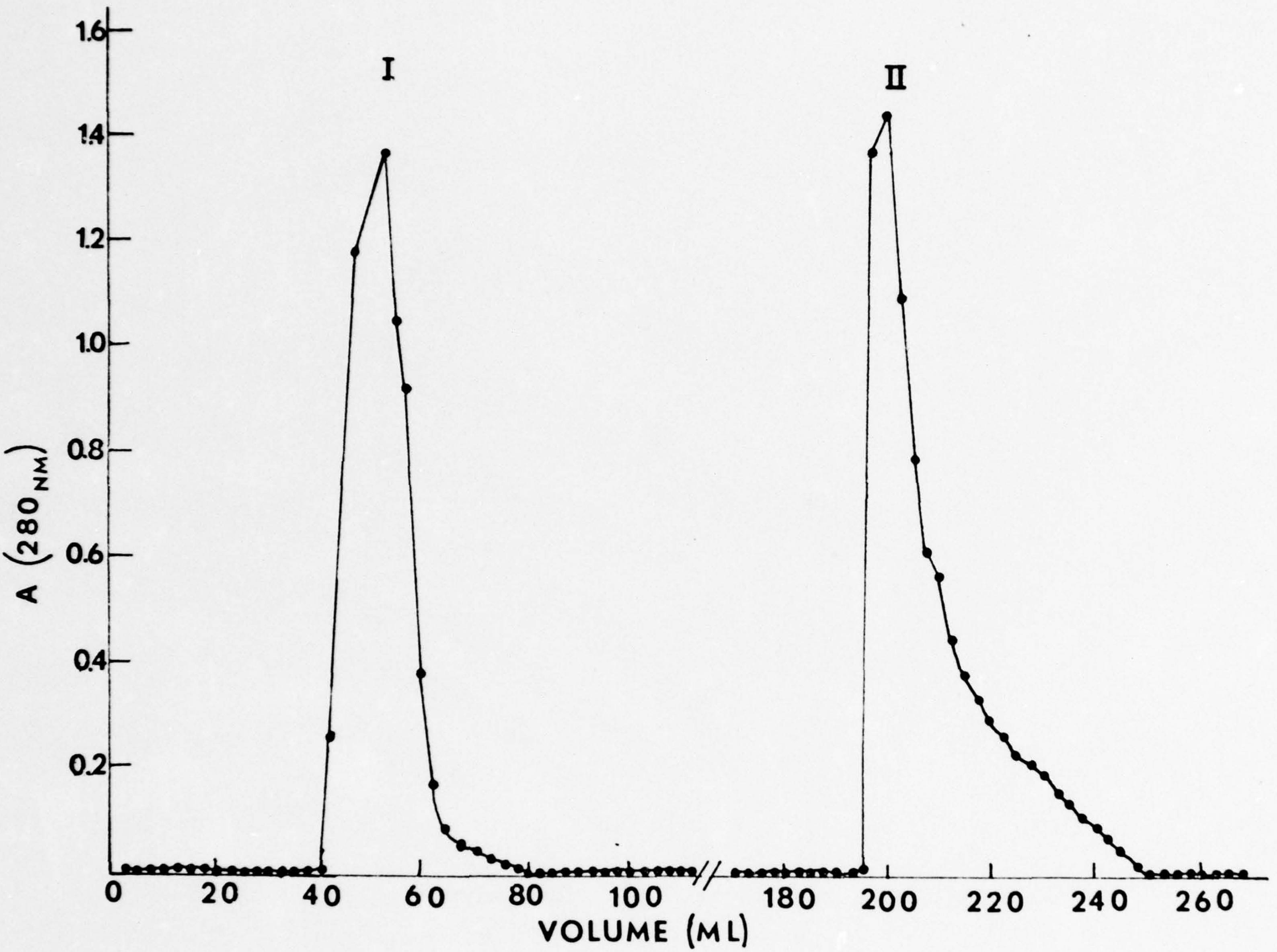

ABSORBANCE 280 nm
0.4
0.3
0.2
0.1
0
5
90
100
110
120
130
140
150
200
FRACTION NUMBER

Fig. 2 Ion exchange chromatography (Dowex AGI (X2) resin) of purified and carboxymethylated glycinin. Column size: 20 x 1 5 cm. Sample: 250 mg modified glycinin in 10 ml of 50 mM TRIS-acetate buffer (pH 8.0) containing 6 M urea. Elution: First elution: in above buffer; second elution in 50 mM acetic acid containing 6 M urea adjusted to pH 4.5 with NaOH. Flow rate: 20 ml/hr. Fraction I: basic subunits of glycinin. Fraction II: acidic subunits of glycinin.


A (280 NM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
180
200
220
240
260
VOLUME (ML)
I
II

the resin are eluted first (peak I). Change of the pH of the elution buffer to 4.5 causes the elution of the acidic subunits (peak II).

SDS-PAGE analysis of DEAE Bio-Gel purified glycinin and fractions I and II eluted from the Dowex AG1 (X2) column is shown in Fig. 3. Two groups of components are distinguishable in regard to a molecular size and these were designated as H and L denoting the heavy and light chains, respectively. In purified glycinin, one L component can be seen. The H components show a major and minor band. From the comparison of the relative mobility (R_f) values of these bands to those of standard proteins, the molecular weight of the major H component was estimated to be 42,000 and that of the minor H component 45,000. The L component has a molecular weight of 19,000. The isolated basic subunits correspond to the L components and the acidic subunits to the H components.

C. Conclusions

We have demonstrated that the use of ion exchange chromatography on Dowex AG1 (X2) column involving a two step pH change of the buffer provides a simple means for the isolation of the acidic and basic subunits of glycinin in bulk quantities (up to 250 mg). The column can be used repetitively after *in situ* regeneration. Thus,

Fig. 3 SDS electrophoresis patterns of: ammonium sulfate purified glycinin (a); DEAE Bio-Gel purified glycinin (b); isolated basic subunits (Fraction I) of glycinin (c); and isolated acidic subunits (Fraction II) of glycinin (d). H and L indicate the position of the heavy (acidic) and light (basic) subunits of glycinin, respectively.

−

H

L

+

a b c d

sufficient quantity of the two types of subunits can be obtained for further fractionation and characterization.

The use of a discontinuous buffer system in SDS-PAGE was used to allow high resolution separation of the subunits. However, the molecular weight of the L component is slightly lower than that reported by Catsimpoolas et al. (3) and Kitamura and Shibasaki (4) using a continuous pH buffer system and in closer agreement to that reported by Badley et al. (5). The major H component exhibits much higher molecular weight by the present technique than previously reported (3-5). However, the molecular weight on the minor H component is in agreement with previously reported values (3,4). These results lead us to suspect that the subunits of glycinin behave abnormally in SDS-PAGE and therefore molecular weight estimation can be considered at best very approximate. This condition does not allow simple arithmetic determination of the number of polypeptide chains from the molecular weight of the protein (5,10) and its subunits. Other methods will have to be employed for comparison. Further work is also necessary to clarify the nature of the minor H component which Kitamura and Shibasaki (4) consider it to be one of the acidic subunits of glycinin.

D. Experimental

The soybeans used were Corosoy variety grown in 1973 and stored at 25°C. The seeds were cracked, dehulled, and ground. The material was defatted with hexane (b.p. 70°C) in a Soxhlet apparatus for 48 hrs. and subsequently desolventized at room temperature.

Glycinin was prepared as described by Wolf *et al.* (8). Briefly, this consisted of extraction of defatted flakes with water (flake:water ratio - 1:5) at 25°C. Subsequently the protein was precipitated in the cold overnight (4°C) and centrifuged to obtain the cold insoluble fraction. The protein in this fraction was precipitated twice with ammonium sulfate (pH 7.6) between 51 and 66% of saturation followed by acid precipitation at pH 4.0, and further precipitation between 26 and 40% saturation with ammonium sulfate.

Chromatographic fractionation of the purified protein was performed on a 4 x 40 cm DEAE Bio-Gel anion exchange column (100-200 mesh) (Bio-Rad Laboratories, Richmond, California) equilibrated with a pH 7.6 phosphate buffer (0.032 M K_2PHO_4, 0.0026 M KH_2PO_4) made 0.1 M in sodium chloride and 0.01 M in mercaptoethanol. Ionic strength elution was carried out by addition of 1M NaCl to a mixing chamber containing 400 ml of the standard buffer.

Detection of the protein fractions was by absorbance at 280 nm. The pooled glycinin fractions were dialyzed against water and freeze dried.

The purified protein (250 mg) was dissolved in 25 ml of 1 M TRIS-HCl, pH 8.7 made 6 M in guanidine hydrochloride and 1 mM EDTA. The solution was flushed with nitrogen for 15 min., then 0.25 ml mercapto-ethanol was added. The solution was incubated at 37°C for 5 hrs. After the addition of iodoacetamide (1.2 gm) the reaction was allowed to proceed in the dark. When a negative nitroprusside test was obtained (about 15 min.), the pritein was dialyzed against water and freeze dried.

Separation of the modified glycinin into acidic and basic subunits was performed on a Dowex AGI (X2) resin (7). Briefly, the resin was washed exhaustively in a Buchner funnel with 2N NaOH, distilled water, 4 N acetic acid, distilled water, and 0.05 M TRIS-acetate buffer, pH 8.0. Just before use the resin was equilibrated on a 1.5 x 20 cm Pharmacia column with 0.05 M TRIS-acetate buffer (pH 8.0) containing 6M urea. The second solution used for elution was

0.05 N acetic acid containing 6 M urea adjusted to pH 4.5 with NaOH. Fractions (2.5 ml) were collected at a rate of 30 ml per hr. The eluate was monitored at 280 nm and 254 nm with an ISCO (Lincoln, Nebraska) Type 6 optical unit and an ISCO UA5 absorbance monitor.

SDS electrophoresis in a discontinuous buffer system was performed according to the method described below. A Bio-Rad Model 221 slab gel electrophoresis cell was used. The polyacrylamide slab (100 mm x 140 mm x 1.5 mm) was prepared as follows: buffers used, 1) ZETA buffer (pH 8.89) containing 6.00 gm glycine, 9.12 gm TRIS, 2.00 gm sodium dodecyl sulfate (SDS), to 2 liters with H_2O, 2) GAMMA buffer (pH 8.92) containing 28.92 ml 1 N HCl, 11.47 gm TRIS, to 100 ml H_2O, 3) BETA buffer (pH 6.44) containing 10.11 ml 1M H_3PO_4, 1.92 gm TRIS, to 100 ml with H_2O. The acrylamide solution used for the separating gel contained 0.6 gm N,N'-methylenebisacrylamide (BIS), 11.4 gm acrylamide (12% T, 5% C) to 50 ml H_2O. The stacking gel consisted of 0.313 gm BIS, 2.817 gm acrylamide (3.13% T, 10% C), to 50 ml with H_2O. The catalyst solution contained 0.03 gm potassium persulfate,

0.001 gm riboflavin, to 100 ml with H_2O. Both separating and stacking gels contained 0.1% SDS. The samples were dissolved in a solution containing 6 M urea, 0.3% SDS, and 10 mM dithiothreitol, and made to volume with ZETA buffer. Bromophenol blue was added as a tracking dye. Sample solutions (1 mg/ml) were incubated at 50°C for 30 min. just prior to sample application. The separating gel was made by mixing 2 parts separating solution, 1 part GAMMA buffer, and 1 part catalyst. This solution was degassed at 15 mm Hg for 10 min. at which time SDS (0.1%) and 25 µl of TEMED were added. The slab was poured and allowed to polymerize under uniform fluorescent light for 45 min. The stacking gel was made by mixing 2 parts stacking solution, 1 part BETA buffer, and 1 part catalyst. The solution was degassed at 15 mm Hg. SDS (0.1%) and 12 µl TEMED was then added. Electrophoresis of 25 µg samples was carried out for approximately 17 hours at 12 mA constant current (20°C). Prior to staining the SDS was removed by fixation for 24 hours in a Bio-Rad Model 222 dual diffusion destainer. The

fixation solution consisted of equal parts of methanol and 20% acetic acid. Subsequently, the slab was stained with 1% Coomassie blue in 7% acetic acid and destained by diffusion in 7% acetic acid, and 30% methanol.

The ZETA, BETA and GAMMA buffers (system No. 2860) were designed by a computer program [1]. Molecular weight determination was performed by another computer system [12] from relative mobility (R_f) values of unknowns versus standard proteins.

ACKNOWLEDGEMENT

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II. Disulfide and Sulfhydryl Groups

A. Introduction

Extensive studies exist at present on the structure of glycinin (1-12). However, the number of sulfhydryl (SH) and disulfide (SS) groups in glycinin is not known, although these moieties have been implicated in the formation of SS polymers (4), heat denaturation (13,14), and intersubunit association of the protein (11, 12, 15). In view of the involvement of the SS bonds in the stabilization of the structure of glycinin and in its denaturation, we have attempted to explore quantitatively their presence and reactivity in urea solutions and alkaline environment. The SS cleaving reagent dithiothreitol (16) was used to measure spectrophotometrically the number and reactivity of SS bonds in urea whereas p-hydroxymercuribenzoate (17) was employed to quantitate SH groups.

B. Experimental

1. Materials

Dithiothreitol (DTT), p-hydroxymercuribenzoate (PHMB) and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo. Ultra pure urea was purchased from Schwarz/Mann Co., Orangeburg, N.Y. All other chemicals were of reagent grade. Glycinin was prepared as previously described (4,18).

Spectrophotometric measurements were performed with a Beckman Model 25 double beam recording spectrophotometer. Matched quartz cuvettes of 1 cm path length were numbered and used in the same sequence for all experiments. A London Radiometer PHM 62 pH meter was used for all pH adjustments.

2. SS Bonds

The assay for the measurement of SS bonds depends on the spectrophotometric quantitation of oxidized DTT (310 nm) produced from the cleavage of SS groups in the protein by the DTT reagent (16). Appropriate blanks have to be provided to correct for (a) possible oxidation of DTT and (b) possible increase of 310 nm absorbance due to scattering from the denatured protein solution. Therefore, the following procedure was employed:

Preparation of solutions

(a) Stock buffer solution: 0.05 potassium

phosphate buffer pH 7.6 made 10^{-3} M in EDTA.

(b) Buffer-Urea (e.g. 8M) solution: Urea (4.8g) dissolved in 10 ml stock buffer solution; prepared fresh just before the assay.

(c) DTT solution (0.05 M): DTT (7.7 mg) dissolved in 0.1 ml stock buffer solution; prepared fresh.

(d) Protein-buffer-urea solution: 10 mg protein dissolved in 1.5 ml buffer-urea solution. Centrifuge at 2,000 g for 15 min. to obtain clear solution for spectrophotometric studies. Protein concentration is determined spectrophotometrically.

All of the solutions placed in the cuvettes are flushed with nitrogen. Urea denaturation of glycinin takes place within a few seconds. However, in this procedure approximately 30 min of preincubation time of the protein with urea was allowed to insure maximum unfolding. For the estimation of the protein concentration, 100 μl aliquot of the protein-buffer-urea solution was mixed with 1.5 ml of the buffer-urea solution and the absorbance at 280 nm was measured against buffer-urea solution as reference. An $E^{1\%}_{1cm}$ value of 8.2 was used to obtain the concentration of glycinin.

Possible changes in light scattering at 310 nm were measured by recording the absorbance of the protein-buffer-urea solution against the buffer-urea solution.

Corrections were made by subtracting this absorbance from that obtained in the presence of DTT. The test solution contained 1.2 ml of the protein-buffer-solution mixed with 10 μl of the DTT solution. The reference was 1.2 ml of the buffer-urea solution mixed with 10 μl of the DTT solution. The absorbance at 310 nm was recorded as a function of time immediately after the addition of DTT. Estimation of the number of SS bonds was performed as described previously (16). Five experiments were performed for each molar concentration of urea.

3. SH Groups

The method described by Boyer (17) was used to measure SH groups in glycinin. This involves spectrophotometric measurement of mercaptide formation employing p-hydroxymercuribenzoate as reagent. The following solutions were prepared:

(e) Stock buffer solution: 0.05 M potassium phosphate buffer pH 7.6.

(f) Buffer-6M urea solution: Urea (3.6 g) dissolved in 10 ml of the stock buffer solution.

(g) PHMB solution (1.8×10^{-3} M): PHMB (6.6 mg) solubilized in minimum 0.1 N KOH and made to 10 ml with stock buffer; prepared fresh.

(h) Protein-buffer-urea solution: 3 mg glycinin dissolved in 3 ml of the buffer-urea solution and centrifuged at 2000 g for 15 min to obtain a clear supernatant. Protein concentration was determined spectrophotometrically as above.

The absorbance at 250 nm due to the protein was measured by reading 1.2 ml of the protein-buffer-urea solution against buffer-urea solution as blank. Subsequently, 10 μl of the PHMB solution were added both to the blank and sample and the increase in absorbance at 250 nm was recorded. A molar extinction coefficient of 7600 for mercaptide formation was used for estimating the number of available SH groups.

In the case of alkali (1.0 N KOH) titration, the difference in absorbance (ΔA) of the protein solution treated with the necessary volume of alkali to obtain a certain pH value versus the protein solution diluted with the same volume of pH 7.6 buffer is estimated at 250 nm. This is necessary in order to correct for the increase in the 250 nm absorbance due to the ionization of tyrosine groups at higher pH values. As an example, 1.2 ml of the protein-buffer-urea solution is treated with a volume (e.g. 0.75 ml) of 1N KOH to bring the pH to 11.9. The blank contains 1.2 ml of the protein-buffer-urea

solution plus 0.75 ml of the stock buffer solution. The ΔA at 250 nm is subsequently subtracted from the ΔA value of the test solution. The test solution contains 1.2 ml of the protein-buffer-urea solution, 0.75 ml 1N KOH and 10 μl PHMB solution and is read against a blank containing 1.2 ml of the protein-buffer-urea solution, 0.75 ml buffer solution and 10 μl PHMB solution. Experiments as above were performed between pH 7.6 and 13.0. The final calculations on the number of SH groups take into consideration the dilution factor due to the addition of alkali.

C. Results and Conclusions

1. Disulfide Bonds

The effect of varying concentrations of urea on the number and rate of cleavage of SS bonds in glycinin by DTT is shown in Fig. 1. A maximum of 20 SS bonds were found using 8 M urea concentration. A lower number of SS bonds were reactive at 4 M and 6 M urea whereas a dramatic decrease was observed at 2 M urea concentration. The rate of the reaction was demonstrated to follow pseudo first order kinetics as illustrated in Fig. 2. A plot of ln (dx/dt) versus time produced the reaction rate constant (k_e) for each urea concentration. When the reaction rate constants were plotted as a function of urea molarity, a linear relationship was obtained (Fig. 3).

The data presented above suggest that the majority

Fig. 1. The time course of glycinin SS bond cleavage by dithiothreitol in various urea concentrations (▲ 2M urea, △ 4M urea, ● 6M urea, o 8M urea).

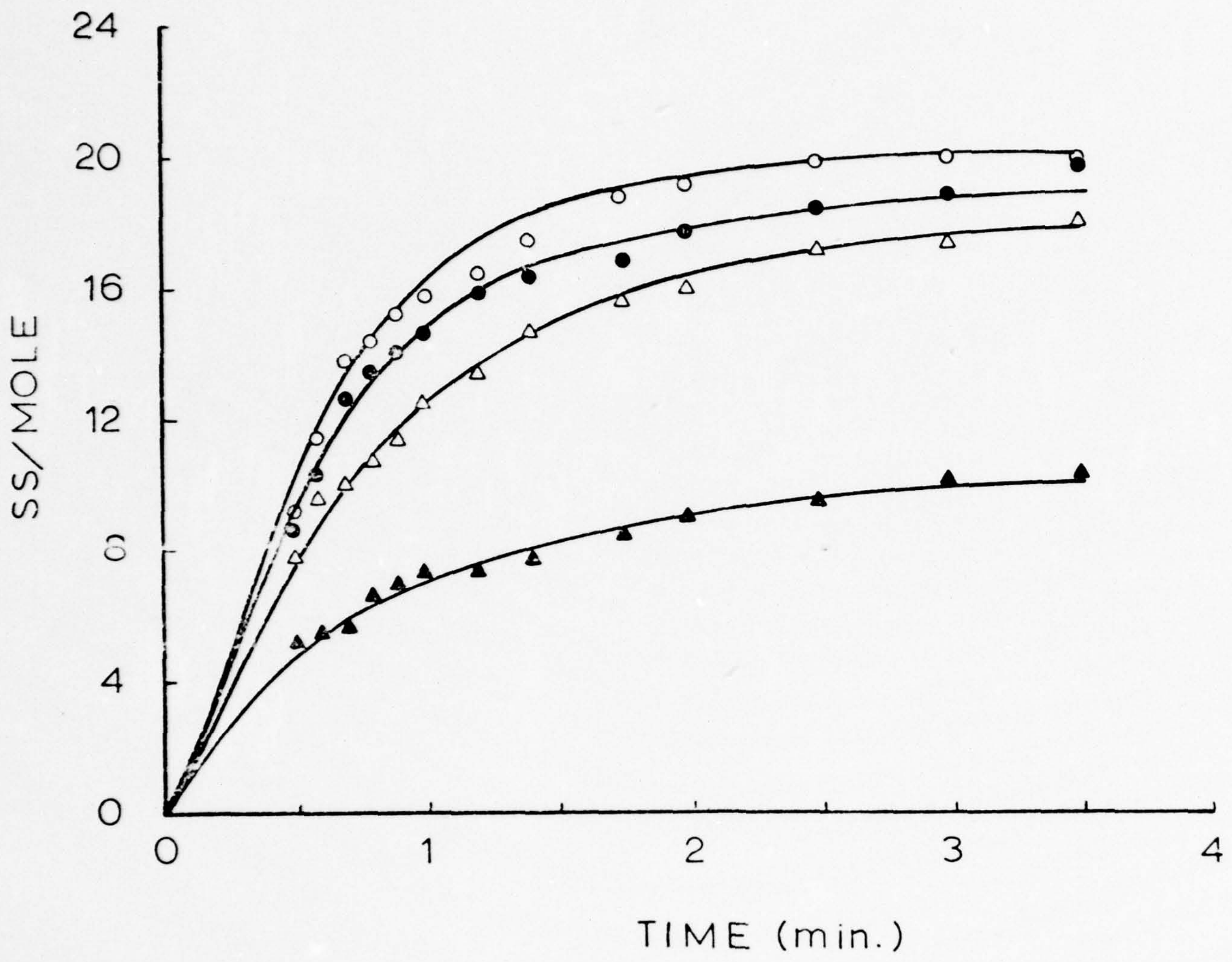

SS/MOLE
24
20
16
12
8
4
0
0
1
2
3
4
TIME (min.)

Fig. 2. Typical pseudo first order reaction plot of SS bond cleavage by dithiothreitol (X = number of disulfide bonds per mole glycinin).

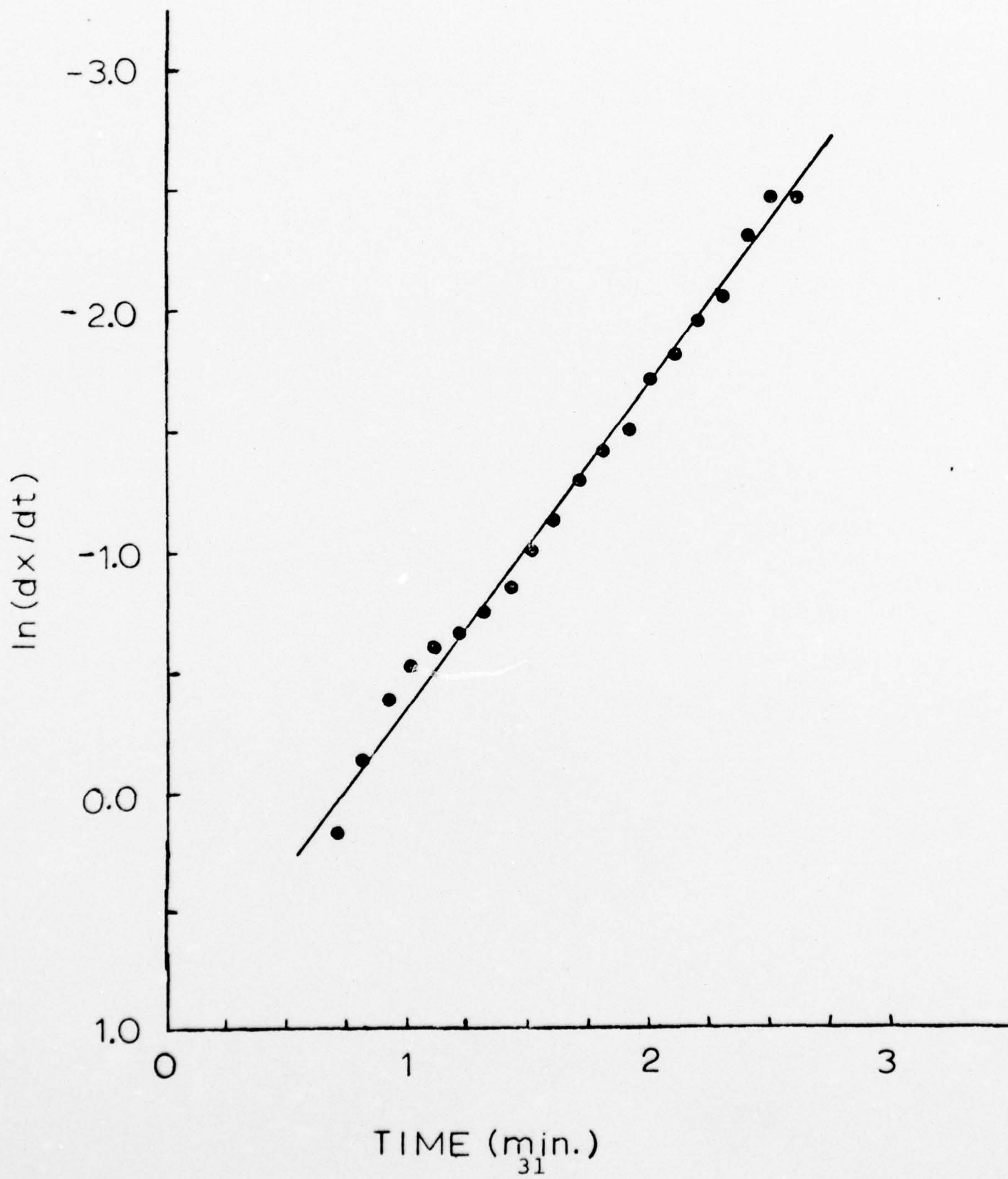
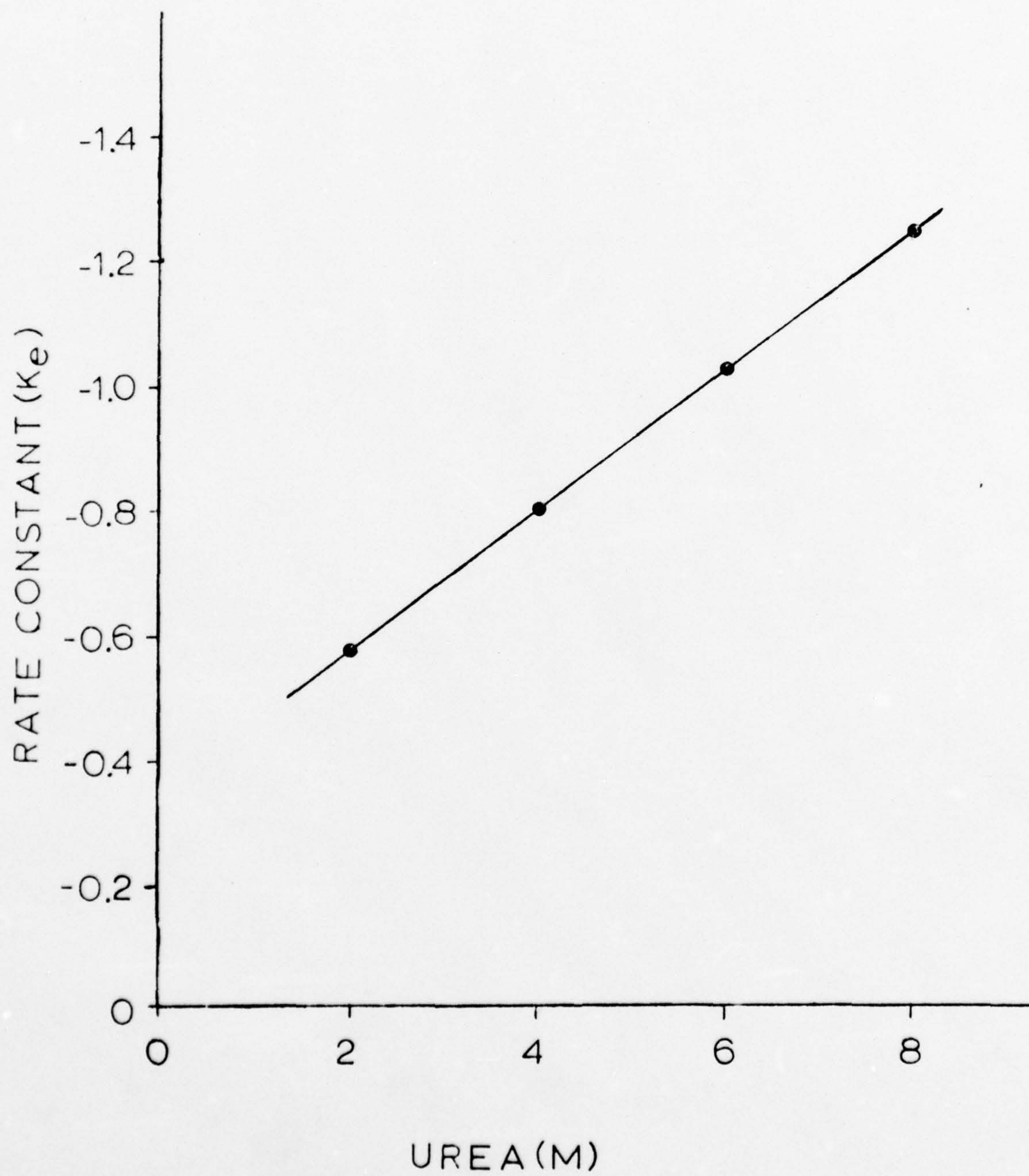

-3.0
-2.0
-1.0
0.0
1.0
ln(dx/dt)
0
1
2
3
TIME (min.)

Fig. 3. Pseudo first order rate constants of SS bond cleavage at varying molar concentrations of urea.


-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0
RATE CONSTANT (K_e)
0
2
4
6
8
UREA (M)

of the disulfide bonds in glycinin are buried in the interior of the protein molecule and become available upon unfolding of the protein with urea. Data in the absence of urea could not be obtained accurately because of light scattering interference due to the high concentrations of protein employed for the spectrophotometric measurements. These problems were alleviated by the presence of urea in the solutions. The finding that glycinin contains at least 20 SS bonds per molecule (320,000 daltons) agrees well with amino acid analysis data of Catsimpoolas *et al.* (8) who found 44 ± 6 half cystine residues per mole of glycinin estimated on the basis of a 350,000 daltons molecular weight. Using the same assumption (i.e. 350,000 MW) glycinin should contain approximately 21.8 SS bonds per molecule plus 1.8 SH groups (see below) thus, producing 45.4 half cystine residues.

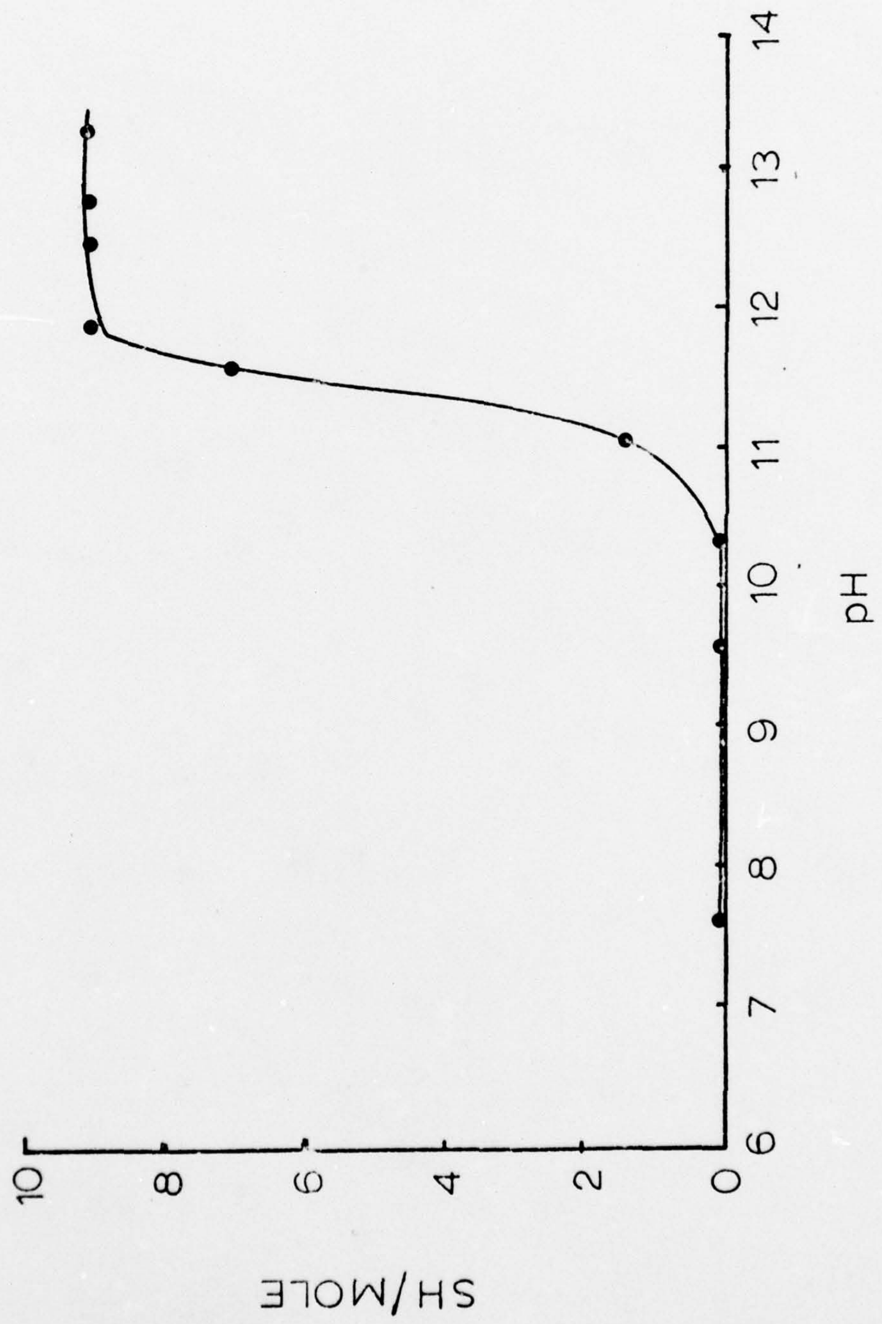
The observed increase in the reaction rate of SS cleavage in the presence of increasing concentrations of urea could be explained as follows. Glycinin may contain several areas of different structural compactness. Upon addition of urea and depending on its concentration only certain regions become unfolded. DTT may attack the SS bonds of the "loose" regions until the maximum number of SS bonds is cleaved. Increased concentration of urea may "loosen up" more structural regions and thus, make available

more SS bonds for interaction. However, the increase in the reaction rate constant with increasing urea concentration may be due to alteration of the "reactivity" of SS bonds related to steric factors in the neighborhood of the cystine molecules.

2. Sulfhydryl Groups

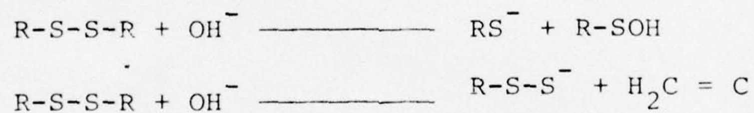
Determination of the sulfhydryl groups of glycinin with PHMB as a function of pH is depicted in Fig. 4. No detectable SH groups were found up to pH 10.5 Above pH 11.0, SH groups appear which reach a maximum of 9.2 groups per mole of glycinin (320.000 MW) at pH 11.9. Thus, there appears to be a narrow pH region where SH groups are present. It was of interest to know if these groups are buried in the native protein molecule being exposed during alkaline denaturation, or are the result of disulfide bond cleavage by the alkali. In a control experiment where glycinin was exposed to 6 M urea and PHMB only 1.7 SH groups per mole of protein could be detected. It was, therefore, concluded that the majority of the SH groups are formed by OH^- scission of exposed SS bonds. The pH effect on the unfolding of glycinin has been studied previously (8) by spectrophotometric titration of tyrosine phenoxy groups. The two curves (Fig. 4 present paper and Fig. 3, Ref. 8) of SH group formation and tyrosine exposure as a function of pH closely resemble each other. The new information

Fig. 4. Reactivity of sulfhydryl groups in glycinin treated with alkali at the indicated pH.


SH/MOLE
10
8
6
4
2
0
6
7
8
9
10
11
12
13
14
pH

that has been obtained from these studies is that alkaline denaturation of glycinin above pH 11.0 also involves cleavage of some susceptible SS bonds.

The effect of OH^- on the SS bonds of proteins has been discussed previously (see Ref. 19) and may involve the formation of either an RSOH moiety which can be further oxidized to cysteic acid, or the generation of a dehydroalamine residue which by interaction with lysine can form lysinoalamine at high pH. The two schemes of cleavage can be illustrated as follows:



$$R\text{-}S\text{-}S\text{-}R + OH^- \longrightarrow RS^- + R\text{-}SOH$$

$$R\text{-}S\text{-}S\text{-}R + OH^- \longrightarrow R\text{-}S\text{-}S^- + H_2C = C$$

From a practical standpoint, some of the above interactions may take place during the first step (i.e. alkaline denaturation) of soybean protein fiber formation (20). In this respect, the suggested (20) sulfhydryl-disulfide interchange may indeed be involved in the mechanism of protein cross-linking in spun fibers. However, Kelley and Pressey (20) did not predict the possible formation of new SH groups by the alkaline treatment of the protein SS groups. The present results provide the first experimental evidence for the presence of buried SS groups in glycinin and the formation of SH groups upon alkaline denaturation.

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PART TWO

I. Summary

Glycinin, the major storage protein of soybeans, was modified by alkali (pH 12.0) and acid (pH 2.0) induced denaturation, and by unfolding in 6M urea followed by cleavage of the disulfide (SS) bonds and blockage of the sulfhydryl (SH) groups to produce CM-Glycinin. The CM-acidic and CM-basic subunits of the protein were also isolated. All the above samples were used to investigate their rate (pH-stat method) and extent (gel filtration analysis) of hydrolysis by trypsin at pH 8.0, 25°C. Native and acid-denatured glycinin and the CM-basic subunits were hydrolyzed slowly over a period of several hours whereas the CM-acidic subunits, alkali-denatured glycinin, and CM-glycinin were attacked at a very fast rate by the enzyme. The molecular weight distribution of the peptide fragments varied in all samples. Characteristic peaks of molecular weight above 30,000, around 7,000 and below 5,000 daltons were observed. In modifications where unfolding of the protein and SS reduction was implicated the peptide fragments were shifted toward the lowest peak(s) i.e. below 5,000 daltons. Glycinin can be hydrolyzed into

large peptide fragments by pepsin within a few minutes, as examined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The acidic subunits of the protein are hydrolyzed faster than the basic subunits. The fast hydrolysis of glycinin by pepsin at pH 2.0 is probably due to the acidic dissociation of the protein into unfolded polypeptide chains. When trypsin was used, glycinin was digested more slowly, but, again the acidic subunits were preferentially cleaved at a faster rate.

Glycinin was also subjected to tryptic hydrolysis at pH 8.0 in the presence of various concentrations of NaCl and examined by uv difference spectroscopy. During hydrolysis, the protein exhibited a typical denaturation "blue shift" of the ultraviolet spectrum. The molar absorptivity difference ($\Delta\varepsilon$) at 291-292nm was used as a relative measure of exposure of buried tryptophan groups. Two types of "reactions were observed leading to a fast (20-30 min) and a slow exposure of chromophoric moieties. Increasing ionic strength significantly diminished the extent of exposure, but enhanced the rate of the fast reaction. Tryptic hydrolysis induced the exposure, but enhanced the rate of the fast reaction. Tryptic hydrolysis induced the exposure of approximately 75% of the tryptophan groups in comparison to 6 M urea

denaturation. This indicates the existence of residual internal structure in the hydrolyzed peptide fragments. It was hypothesized that the fast and slow rates of exposure may reflect hydrolysis of the acidic and basic subunits of glycinin, respectively. Based on these results, a physiological role for the acidic and basic subunits of glycinin in the germinating seed was proposed.

II. Introduction

Soybeans, originating from the Orient, have become an important crop in the United States since the late 1920's, when soybean processing became an established industry. Due to a surplus of agricultural crops at the time, the oil was used for industrial purposes and the meal was used for animal feeds. Recently, soybeans have become a major source of protein, mainly supplemental, due to the high cost of protein sources such as meat. It has become one of the major ingredients in fabricated foods, such as meat analogs.

There exist three basic forms of processed soybean protein used as food ingredients for the variety of soybean protein products now available. They are classified according to their protein content and are

soy flour and grits, protein concentrates, and protein isolates in increasing order of their protein concentration. To take advantage of the functional properties of the various processed proteins, they may be added to food systems for the purpose of emulsification, fat absorption, gelatin and aeration. Also, these three major forms may be further processed before being added to a food system. For example, soy flour may be extruded or isolates may be spun to impart meat-like textures or to simulate fruits, nuts or vegetables. Both of these latter processes involve acid precipitation of the protein and the addition of alkali to the protein.

To understand how the properties of the soybean proteins will change under various processing and enzymatic modification conditions and how their nutritive value may change, it is important to have some knowledge of the physical and chemical properties of the individual proteins. Since the initial step in the production of any of the protein products is the water extraction of defatted soybean flakes, the proteins of interest are the water-soluble ones. Four major fractions are observed in this extract and are designated as 2,7,11 and 15S, based on their ultracentrifugation sedimentation rates (1). The 11S component,

glycinin, provides a suitable material for study since it is the major soybean protein, and can be obtained in homogeneous from (2,3), and its structure is known in considerable detail (4-7). Glycinin has a molecular weight of 350,000 (5) and consists of six acidic and six basic subunits (4) exhibiting molecular weights of 37,2000 and 23,000 respectively (8). The acidic subunits have a higher content of glutamic acid and proline than the basic ones, whereas the basic subunits have larger amounts of the hydrophobic amino acids leucine, tyrosine, phenylalanine, valine and alanine (8). It has been postulated that hydrophobic bonds play an important role in the stabilization of the glycinin subunits (9,10).

In view of the importance of enzymatic hydrolysis studies in understanding (a) the mechanism of degradation of storage proteins during seed germination (b) the digestibility of soybean protein in the gastrointestinal tract and (c) the production of modified soybean proteins for food use, this laboratory has been engaged in the investigation of the enzymatic hydrolysis of glycinin. In the present report, we describe pH-stat, molecular weight distribution, and uv difference spectra studies performed on the products of enzymatic hydrolysis deriving from native and denatured glycinin and its acidic and basic subunits.

III. Experimental

A. Glycinin Isolation

Soybeans Corsoy 73 (U.S.A.) were used as the protein source for the preparation of glycinin. The soybeans were first cracked using a Waring blender, then dehulled. The oil was extracted with hexane in a Soxhlet extractor. Following this, the defatted flakes were ground to a coarse meal. Isolation of glycinin from the meal was achieved by the method of Wolf and Briggs (5). Briefly, this entailed the extraction of the meal (100 g) with water (1 part meal to 5 parts water) for 1 hr. The mixture was then passed through cheese-cloth to remove any large particles. Centrifugation at 2,000 x g at room temperature was used to clarify the aqueous extract, after which the supernatant was incubated at 0-4°C overnight. Following centrifugation at 10,000 x g at 0-4°C, the preciptate was dissolved in a total of 72 ml of phosphate buffer (pH 7.6, μ = 0.4M in NaCl and 0.01 M mercaptoethanol). (The buffer was prepared as follows: 5.66 g K_2HPO_4, 0.354 g KH_2PO_4, 23.36 g NaCl and 0.71 ml of mercaptoethanol were made up to 1.0 l with water.) The protein solution was made 0.11 M (0.10 g) with respect to N-ethylmaleimide and stirred for 30 min. This was then diluted to 17000 ml

with phosphate buffer (pH 7.6, μ = 0.4 M, 0.01 M mercaptoethanol), and adjusted to 51% saturation with $(NH_4)_2SO_4$ (670 g). After stirring for 30 min, the solution was centrifuged at 2,000 x g and the supernatant was adjusted to 66% saturation with $(NH_4)_2SO_4$ (211 g) and then stirred for 30 min. The precipitate obtained upon centrifugation was dissolved in phosphate buffer (pH 7.6, μ = 0.4 M, 0.01 M mercaptoethanol) to a volume of 1.0 l. After adjusting the solution to 66% saturation with $(NH_4)_2SO_4$ (522 g) and stirring for 30 min, it was centrifuged again at 2,000 x g. The precipitate was dissolved in 1.0 M NaCl for 24 hr at 0-4°C. The dialysate, upon warming to room temperature was diluted to 100 ml with 1.0 M NaCl. This was made 10^{-3} M with respect to N-ethylmaleimide (0.0125 g), and stirred for 20-30 min. The pH of this solution was adjusted to 26% saturation with $(NH_4)_2SO_4$ (15 g/100 ml). After stirring for 10 min, the mixture was centrifuged at 10,000 x g and the precipitate was dissolved and dialyzed overnight against phosphate buffer (pH 7.8, μ = 0.1 M in NaCl, 0.01 M mercaptoethanol).

To purify the protein (3), an ion exchange column (Bio-Rad Laboratories, Richmond, CA Bio-Rex Type MT, 2.5 cm x 50 cm) was prepared using DEAE-Sephadex (Pharmacia, Sweden) equilibrated in phosphate buffer

(pH 7.8, μ = 0.1 M, 0.01 M mercaptoethanol). The approximate protein concentration was determined to prevent overloading of the column, by measuring the absorbance of the protein solution at 280 nm. The concentration was then estimated from E = 8.2, where E denotes the absorbance of a 1% protein solution in 1 cm cuvette at 280 nm.

The dialyzed protein solution was eluted from the column with phosphate buffer (0.01 M mercaptoethanol) in a NaCl ionic strength gradient ranging from 0.01 M to 1.0 M in NaCl. Fractions were collected with an ISCO (Lincoln, NB) Model 328 fraction collector and monitored with an ISCO Type 6 Optical Unit, Model UA-5 Absorbance Monitor and Model 1132 Multiplexer-Expander and recorded with an ISCO recorder. The major peak of the uv absorbance profile eluted at approximately 0.7 M NaCl represented purified glycinin.

B. Reaction of Glycinin Sulfhydryl Groups with Iodoacetamide

CM-glycinin, i.e. modified by reduction of the disulfide bonds and blockage of the sulfhydryl (SH) by iodoacetamide, was prepared as follows (11). The purified protein (250 mg) was dissolved in 25 ml of 1 M Tris-HCl, pH 8.7 made 6 M in guanidine HCl and 1 mM

EDTA. The solution was flushed with N_2 for 15 min, then 0.25 ml mercaptoethanol was added. The solution was incubated at 37° for 5 hr. After the addition of iodoacetamide (1.2 g) the reaction was allowed to proceed in the dark. When a negative nitroprusside test was obtained (about 15 min), the protein was dialyzed against H_2O and freeze-dried.

C. Isolation of the Acidic and Basic Subunits of Glycinin

This was performed by an exchange chromatography method described recently (11). Briefly, a Dowex AG1 (X2) ion exchange resin was washed exhaustively in a Buchner funnel with 2N NaOH, H_2O, 4N HOAc, H_2O and 0.05 M Tris-acetate buffer, pH 8.0. Just before use the resin was equilibrated on a 1.5 x 20 cm Pharmacia column with 0.05 M Tris-acetate buffer (pH 8.0) containing 6M urea. CM-glycinin (250 mg) was applied on the column. Elution of the basic subunits was carried out with the above buffer whereas the acidic subunits were subsequently eluted by 0.05 N HOAc containing 6 M urea adjusted to pH 4.5 with NaOH. Fractions (2.5 ml) were collected at a flow rate of 30 ml per hour. Two distinct peaks appear in the uv elution profile which represent the two types of the glycinin subunits. The fractions were dialyzed vs. water to remove the reagents and freeze-dried.

D. pH-Induced Denaturation of Glycinin

Glycinin (10 mg) was added to 10 ml of 0.1 M KCl

solution adjusted to pH 8.0 with NaOH. Subsequently, the pH was adjusted to 2.0 with 0.01 N HCl and the solution was stirred for 15 min. Then 0.01 N NaOH was used to bring the pH back to 8.0. This sample was denoted as acid-denatured glycinin. To prepare alkali-denatured glycinin, the protein (10 mg) was dissolved directly in 0.01 N NaOH, 0.1 M KCl (pH 12.0). The solution was stirred for 15 min and then adjusted to pH 8.0 with HCl. The modified-protein solution was dialyzed vs. water and freeze-dried.

E. Tryptic Hydrolysis - pH-stat

Enzymatic hydrolysis of glycinin, pH-denatured glycinin, CM-acidic, and CM-basic subunits was performed in a London Radiometer pH-stat apparatus. The experimental set-up included a Titrator, Type TTT 60; Titrigraph, Type SBR 3; Autoburette, Type ABU 11; Standard pH meter, Type PHM 62; G202C glass electrode and K401 Calomel electrode. The reaction was carried out in a beaker surrounded by a jacket (Type V525) containing a 1% NaCl solution. Stirring was achieved by a magnetic stirrer and a current of N_2 excluded CO_2 from the reaction vessel. All solutions were prepared from CO_2-free distilled water achieved by boiling distilled water prior to preparing the solutions. All titrations were performed with

0.001 M NaOH as the titrant, at pH 8.0, with an enzyme: protein ratio of 1:100. Trypsin (Miles Laboratories, 36-555) was dissolved in 0.1 M KCl and brought to about pH 8.0 with NaOH. The same solution was used as the reaction solvent.

A typical run was done as follows: 10 ml of the 0.1 M KCl solution adjusted to about pH 8.0 was pipetted into the reaction vessel. To this, 10 mg of protein were added. The pH was then adjusted to pH 8.0 with titrant, with the stirrer and the N_2 stream on. After the pH had stabilized, 0.5 ml of trypsin solution (1 mg/ml) was added with a plastic syringe. The reaction was then allowed to proceed until no further uptake of base was observed. The reaction mixtures were then lyophilized to be used for gel filtration analysis.

F. Analytical Gel Filtration

1. Materials

Sephadex G-50 (superfine) and Blue Dextran were obtained from Pharmacia. Insulin and DNP-alanine (lot #23C-2910) were purchased from Sigma. α-Chymotrypsin (lot #629) was a product of Miles Laboratories. Cytochrome C (82C-7700) and myoglobin (lot #Y3230) were obtained from Schwarz/Mann and ribonuclease (lot #R35C766)

was obtained from Worthington.

The solvent used in the chromatrographic system was a phosphate buffer 0.1 M in NaCl, pH 7.8 that was made 0.02% in sodium azide.

2. Gel chromatography

An LC Cheminert glass column (LC-9MA-13, Laboratory Data Control, Riviera Beach, FL.), 9.0 mm bore and 33 mm length, with inlet and outlet Cheminert fitting was used for these experiments. A sample injection tee was used for the addition of sample to the column, using a plastice syringe and female luer adapter to fill the 100 μl sample loop. A Milton Roy Constametric I metering pump (LDC) was used to pump the buffer at a constant flow rate (0.16 ml/min) from a reservoir through the reference cell of a Schoeffel (Westwood, N.J.) SF 770 Spectroflow Monitor (10 mm path length) to the inlet of the sample injection tee. The eluent from the column was monitored at 220 nm. The LDC Series 3400 Chromatographic Recorder was operated at a constant speed of 2mm/min. An event marker was actuated at the time of sample injection.

The column was packed with the inlet plunger assembly facing down using the pump and an extension to the column (packing reservoir LCR-9M). The column was first filled with buffer, then a slurry of the

Sephadex G-50 equilibrated with buffer was added to the packing reservoir. The head space of the reservoir was then filled with buffer. After attaching the plunger assembly to the top of the packing reservoir, the column was allowed to pack under positive pressure generated by the pump. The packing reservoir was removed after the column had been packed and the outlet assembly was inserted into the column so that the bed support of the plunger assembly touched the column bed. All of the connections were made using teflon tubing and LDC/Cheminert fittings.

3. Column calibration and molecular weight determinations

To calibrate the column, insulin, cytochrome C, ribonuclease, myoglobin and α-chymotrypsin were used. Dextran Blue and DNP-alanine were added to each sample to determine the minimum (t_o) and the maximum (t_i) retention times. The retention time of a particular protein is expressed by t_e. (Retention time is the time interval from the injection of the sample to the peak maximum.) All of the solutions were prepared with standard buffer. For sample injection, 1.0 ml of the protein solution (0.2 mg/ml) was combined with 0.5 ml of Dextran Blue (2.0 mg/ml), 0.5 ml of DNP-alanine

(0.2 mg/ml) and 0.5 ml of buffer. Since a 100 μl sample loop was employed, this resulted in the addition of 8.9 μg of protein to the column. The absorbance scale used was 0-0.2 absorbance units at 220nm.

From the peak maxima, partition coefficients were determined for each marker protein and peptide as $(t_e-t_o)/t_i$ corresponding to $(V_e-V_o)/V_i$ in volume measurements (13). For molecular weight estimation, the data was plotted according to Hjertén[15] substituting $(V_e-V_o)/V_i$ with $(t_e-t_o)/t_i$. The intercept and slope of the straight line were obtained by a least squares treatment of the data. The regression equation was:

$$-\log \sigma = 0.0017\ M^{\frac{2}{3}} + 0.2157.$$

The hydrolysate samples were analyzed at a concentration of 7 mg/2.5 ml or 7 mg/2.0 ml, using an absorbance range of 0-0.1 absorbance units. The data were digitized at 1 min intervals to obtain the height of the distribution. The weight-average molecular weight ($\bar{M}_w$) of a selected distribution was determined with the use of a digital computer program by the method of Catsimpoolas (13).

G. Preparation of Hydrolysates for Electrophoretic Analysis

Enzymatic hydrolysis of isolated glycinin was carried out with pepsin at pH 2.0 at 37°C, 25°C and 4°C and

trypsin at pH 8.0 at 25°C. The peptic hydrolysis was performed in 0.01 N HCl whereas the tryptic hydrolysis involved incubation in a Tris-HCl buffer (pH 8.0, 0.46 M). The enzyme to protein ratio was 1:100 in all the experiments. the concentration of glycinin was 0.1%. To stop the enzyme action an aliquot (100 μl) of the hydrolysate was added to an equal volume of a solution containing 6 M urea, 0.3% SDS and 10 mM dithiothreitol, pH 8.9. The mixture was stored at -6°C until electrophoretic analysis was performed which involved sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuos buffer system (15).

H. SDS-PAGE Electrophoretic Analysis

SDS-PAGE electrophoresis in a discontinuous buffer system was performed according to the method described below. A Bio-Rad Model 221 slab gel electrophoresis cell was used. The polyacrylamide slab (100 mm x 140 mm x 1.5mm) was prepared as follows: buffers used, 1) ZETA buffer (pH 8.89) containing 6.00 gm glycine, 9.12 gm TRIS, 2.00 g, sodium dodecyl sulfate (SDS), to 2 liters with H_2O, 2) GAMMA buffer (pH 8.92) containing 28.92 ml 1 N HCl, 11.47 gm TRIS, to 100 ml H_2O 3) BETA buffer (pH 6.44) containing 10.11 ml 1 M H_3PO_4, 1.92 gm TRIS, to 100 ml with H_2O. The acrylamide solution used for

the separating gel contained 0.6 gm N,N'-methylenebis-acrylamide (BIS), 11.4 gm acrylamide (12% T, 5% C), to 50 ml H_2O. The stacking gel consisted of 0.313 gm BIS, 2.817 gm acrylamide (3.13%T, 10%C), to 50 ml with H_2O. The catalyst solution contained 0.03 gm potassium persulfate, 0.001 gm riboflavin, to 100 ml with H_2O. Both separating and stacking gels contained 0.1% SDS. The samples were dissolved in a solution containing 6 M urea, o.3% SDS, and 10 mM dithiothreitol, and made to volume with ZETA buffer. Bromophenol blue was added as a tracking dye. Sample solutions (1 mg/ml) were incubated at 50°C for 30 min just prior to sample application. The separating gel was made by mixing 2 parts separating solution, 1 part GAMMA buffer, and 1 part catalyst. This solution was degassed at 15 mm Hg for 10 min at which time SDS (0.1%) and 25 µl of TEMED were added. The slab was poured and allowed to polymerize under uniform flourescent light for 45 min. The stacking gel was made by mixing 2 parts stacking solution, 1 part BETA buffer, and 1 part catalyst. The solution was degassed at 15 mm Hg. SDS (0.1%) and 12 µl TEMED were then added. Electrophoresis of 25 µg samples was carried out for approximately 17 hours at 12 mA constant current (20°C). Prior to staining

the SDS was removed by fixation for 24 hours in a Bio-Rad Model 222 dual diffusion destainer. The fixation solution consisted of equal parts of methanol and 20% acetic acid. Subsequently, the slab was stained with 1% Coomassie blue in 7% acetic acid and destained by diffusion in 7% acetic acid, and 30% methanol.

The ZETA, BETA and GAMMA buffers (system No. 2860) were designed by a computer program (15). Molecular weight determination was performed by another computer system (16) from relative mobility (R_f) values of unknowns versus standard proteins.

I. Ultraviolet Difference Spectra

1. Spectrophotometric procedure

A Beckman Model 25 double beam recording spectrophotometer was used for these experiments. A tandem arrangement of quartz cuvettes was set-up as follows: The "reference" position contained one cuvette with 2.1 ml of the buffer (TRIS-HCl pH 8.0, 0.046 M) and another with 2 ml of the glycinin solution (0.1%) in the buffer plus 0.1 ml of the enzyme solution (0.02%) in the buffer. The enzyme solution was added at "zero" time. The "sample" position contained one cuvette with 2.0 ml of the protein solution in buffer plus 0.1 ml of

the buffer and the other 2.0 ml of the buffer plus 0.1 ml of the enzyme solution in buffer. The placement of the reaction (hydrolysis) mixture of protein plus enzyme in the "reference" position was done to obtain "positive" values of absorbance changes (ΔA) rather than negative. In some experiments the TRIS-HCl buffer was made 0.04 M, 0.07 M and 0.1 M in regard to NaCl. Ultraviolet absorption difference spectra were measured every 5.0 min after the addition of the enzyme to the protein. The scanning wavelength ranged from 220nm to 350 nm.

2. Computations

The rate of exposure of chromophoric groups at a specific wavelength as measured by absorbance differences (ΔA) can be described by combination of a first rate equation with its integrated form:

$$\ln \frac{d(\Delta A)}{dt} = \ln (\Delta A)_o K_e - K_e t$$

where: K is the rate constant of exposure of chromophoric groups; $d(\Delta A)/dt$ the rate of absorbance difference at time t; and $(\Delta A)_o$ is the total absorbance difference observed.

A plot of the natural logarithm of the exposure rate - from the first order equation - against time should yield a straight line with slope equal to K and intercept

to $\ln(\Delta A)_o K_e$.

To perform all the above calculations, it is necessary to compute the first derivative d(A)/dt vs. t from the original experimental data of A vs. t. This can be carried out by computing the orthogonal linear component y'(d(A)/dt) by using a five-point third-order polynomial fitting technique from:

$$y' = (-2(\Delta A)_{i-2} - (\Delta A)_{i-1} + \emptyset(A)_i + 2(\Delta A)_{i+2})/10$$

where $(\Delta A)_{i\pm n}$ represents the observed ΔA values of a five data-point moving box.

In these experiments in order to compare results with our previous studies (17) all ΔA values were converted to molar absorptivity differences $\Delta\varepsilon$ before computation of the results in terms of kinetics. The molecular weight of glycinin was assumed to be 320,000 (4,10). The rate constant for the slow exposure was designated as K_s and that of the fast as K_f.

IV. Results

A. pH-Stat Studies

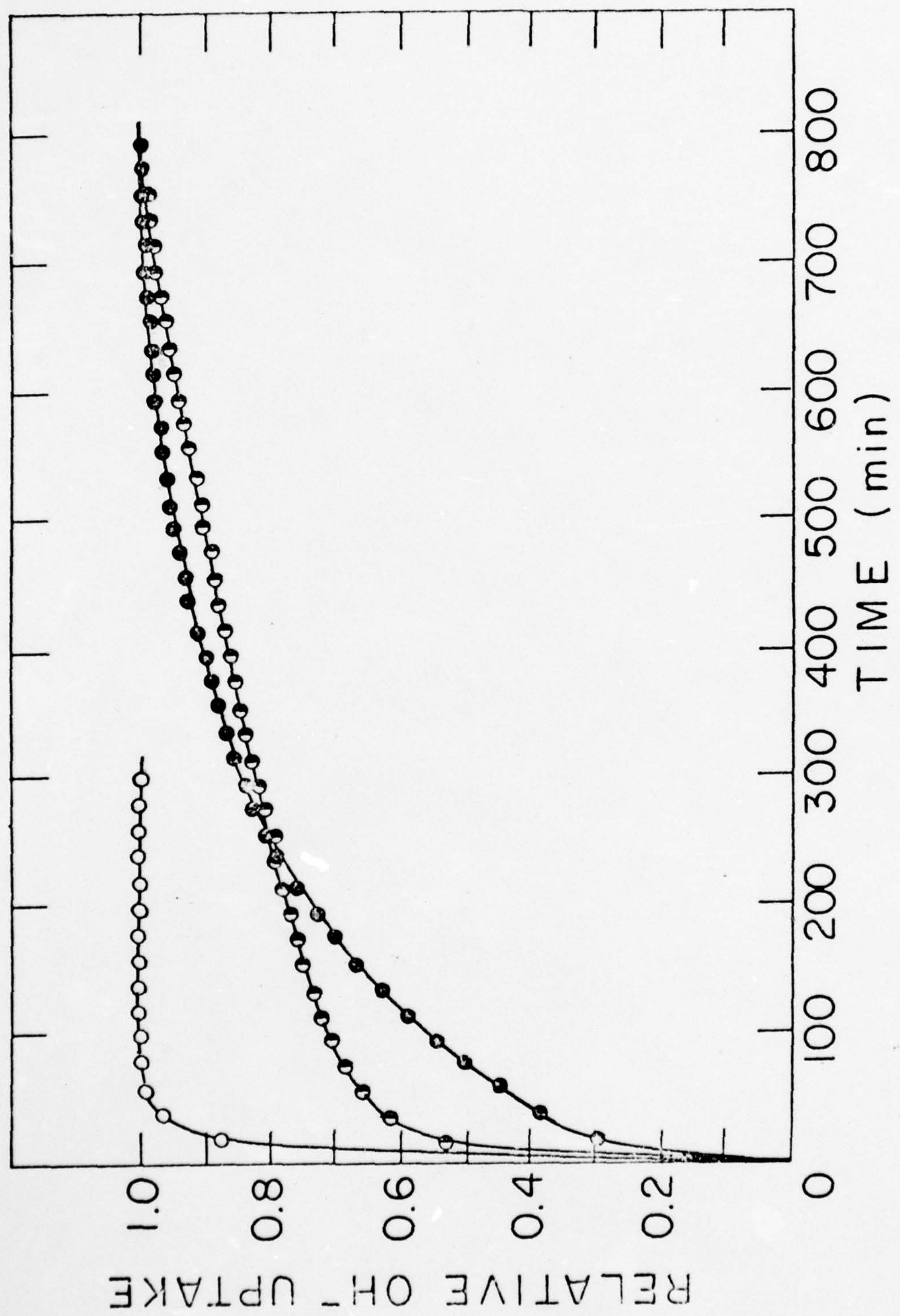
The pH-stat procedure was used to monitor the base (OH^-) uptake during the tryptic hydrolysis of glycinin and its subunits at pH 8.0, 25°C. Three samples of glycinin were examined: native, denatured by acid

(pH 2.0); and denatured by alkali (pH 12.0). A representative reaction course for these three samples is shown in Figure 1. The hydrolysis of glycinin denatured by alkali was completed in 90 min. (Complete is defined as no further base uptake). Glycinin in its native state and acid-denatured glycinin both required 13-20 hr for hydrolysis to be completed. However, on inspection of the reaction curves, it was observed that the rate of hydrolysis of the acid-denatured glycinin was faster at the start of the reaction and slower towards the end in comparison to native glycinin.

CM-glycinin and its isolated CM-acidic and CM-basic subunits hydrolyzed by trypsin at pH 8.0 and followed by the pH stat procedure produced representative reaction curves as shown in Figure 2. The rate of hydrolysis of the CM-acidic subunits and CM-glycinin are comparable, whereas the hydrolysis of the CM-basic subunits required a longer length of time for completion. In comparison to the untreated glycinin, the CM-glycinin required about 1/3 less time for hydrolysis to be completed.

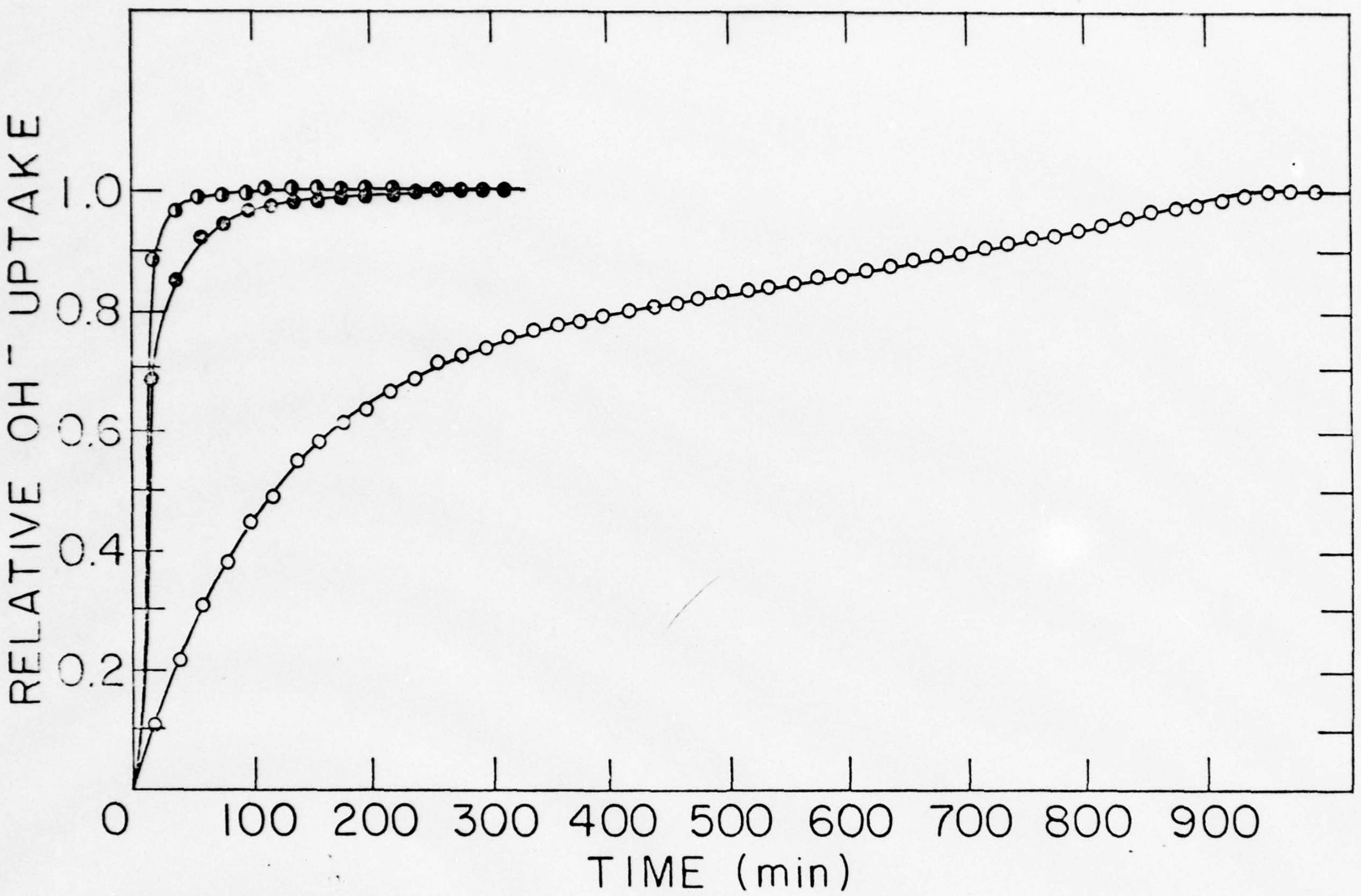
If all samples are compared, the alkali-denatured glycinin and the isolated CM-acidic subunits exhibit the faster rate of hydrolysis followed very closely by CM-glycinin. The native and acid-denatured glycinin show

Figure 1. Representative pH-stat curves of the enzymatic hydrolysis of glycinin, acid-denatured glycinin (pH 2.0), and alkali-denatured glycinin (pH 12.0) by trypsin at pH 8.0, 25°C. The values for the extent of hydrolysis have been normalized to the maximum value for each curve. Key: ○—○ = alkali-denatured glycinin; ◑—◑ = acid-denatured glycinin; ●—● = native glycinin.



RELATIVE OH⁻ UPTAKE
1.0
0.8
0.6
0.4
0.2
0
100
200
300
400
500
600
700
800
TIME (min)

Figure 2. Representative pH-stat curves of the enzymatic hydrolysis of CM-glycinin and its isolated acidic and basic subunits by trypsin at pH 8.0, 25°C. The values for the extent of hydrolysis have been normalized to the maximum value for each curve. Key: ◑—◑ = CM-acidic subunits; ●—● = CM-glycinin; ○—○ = CM-basic subunits.



RELATIVE OH⁻ UPTAKE
1.0
0.8
0.6
0.4
0.2
0
100
200
300
400
500
600
700
800
900
TIME (min)

intermediate rates of hydrolysis whereas the CM-basic subunits are hydrolyzed with the slowest rate.

B. Molecular Weight Analysis by Gel Filtration

Native glycinin hydrolyzed by trypsin (pH 8.0, 25°C) for various time intervals was analyzed by gel filtration chromatography with the results shown in Figure 3 and Table 1. Three discernible peaks could be distinguished with apparent molecular weights of >30,000, 6,500-9,500, and <5,000 daltons. The area of these peaks was estimated and the values were tabulated in Table 1. It is evident that the high molecular weight peak No. 1 is decreased with the time of hydrolysis with a corresponding increase of the low molecular weight peptides (<5,000, peak No.3). The proportional area of peak No.3). The proportional area of peak No. 2 did not change appreciably in comparison to the other peaks.

Alkali denatured (pH 2.0) and acid denatured (pH 12.0) glycinin upon completion of trypsin hydrolysis i.e. at 1.3 and 19 hr, respectively exhibited the gel filtration patterns shown in Figure 4. The areas of the peaks are tabulated in Table 2. It is evident that in both samples there is a negligible amount of high molecular weight components (peak I) and considerable amount of peak II. However, two peaks (IIIa and IIIb)

Figure 3. Gel filtration chromatographic analysis of glycinin hydrolyzed by trypsin at pH 8.0 at various time intervals. The values of 220nm absorbance are normalized relative to the highest peak of each sample for comparison.

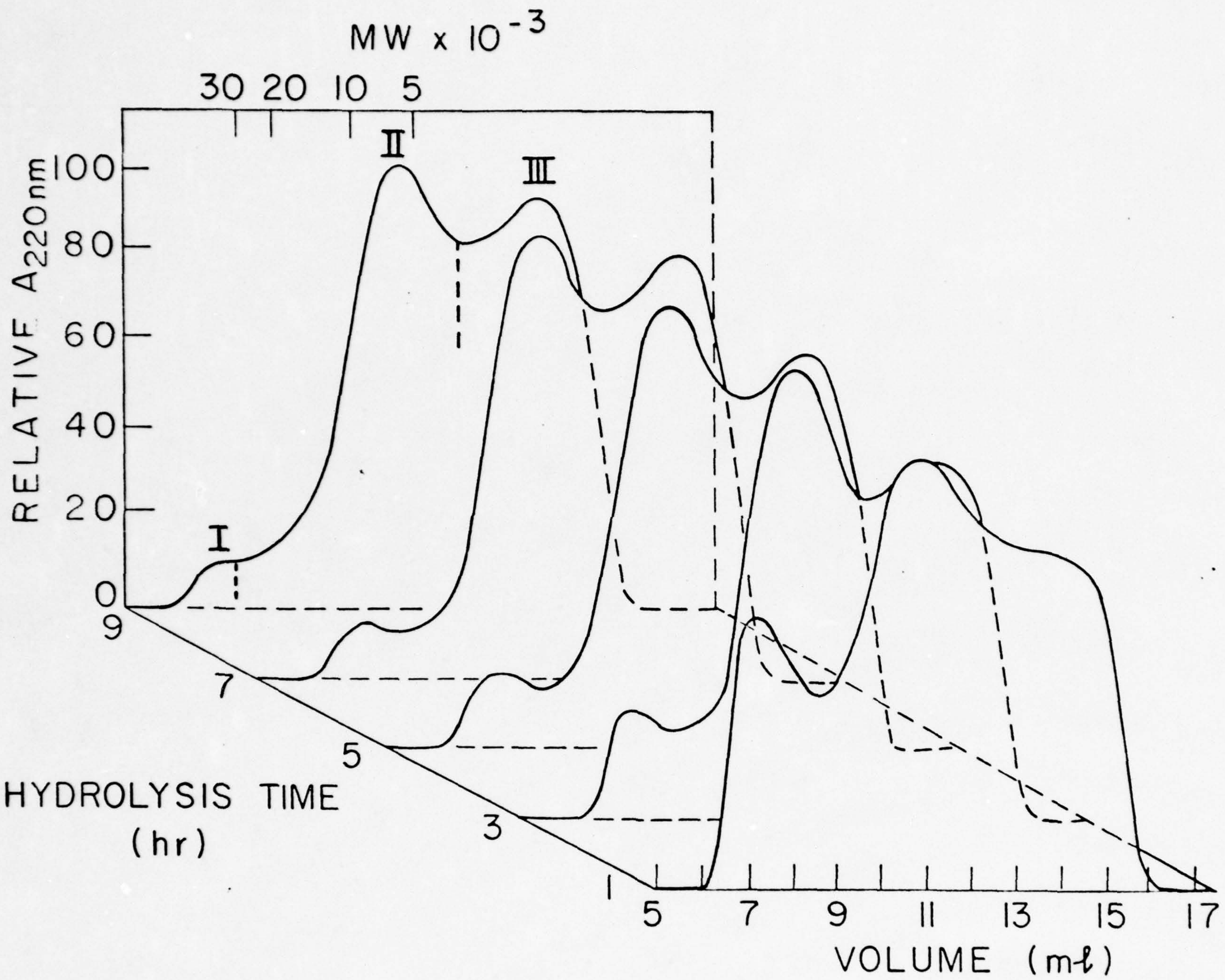
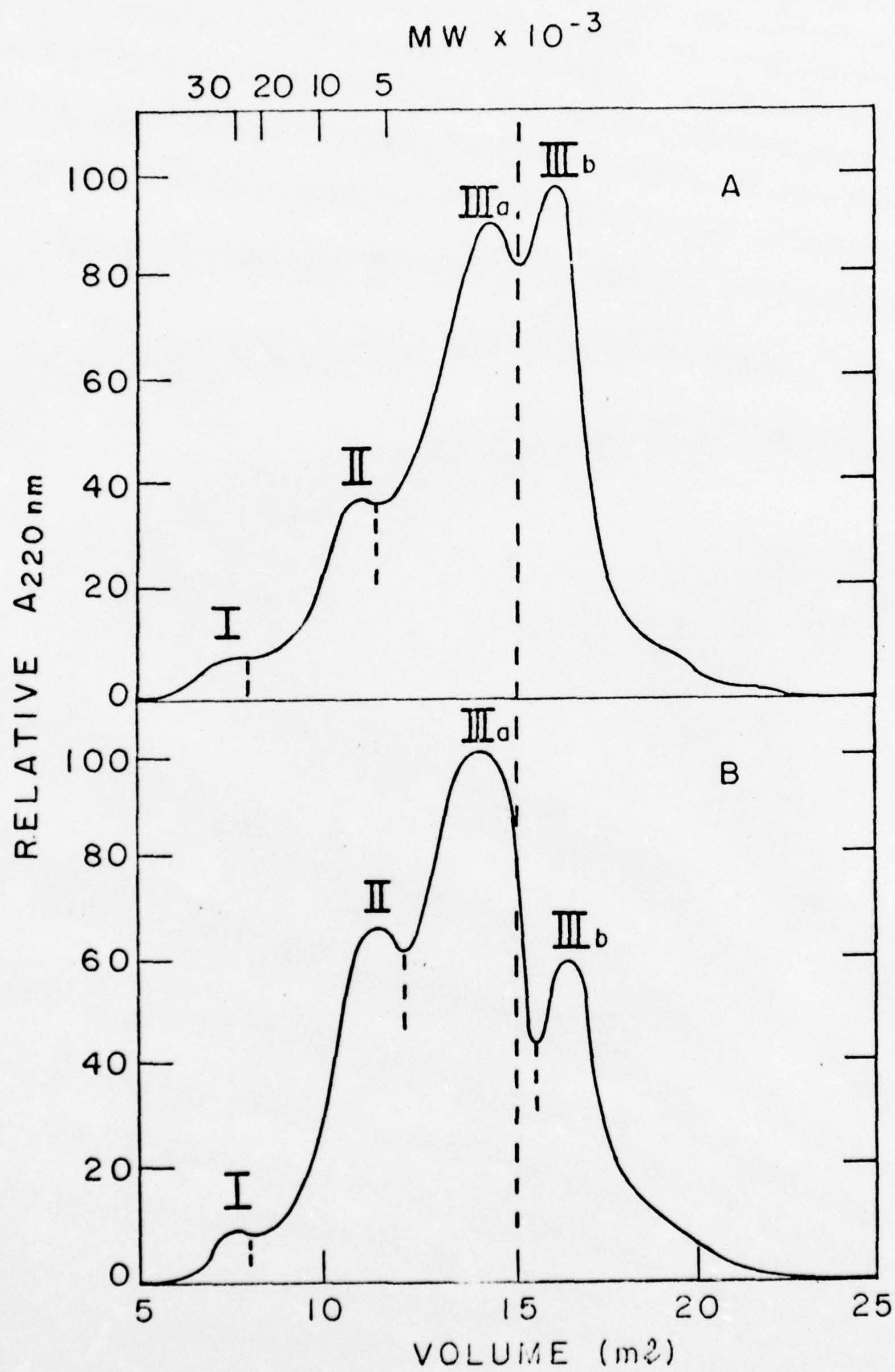

MW x 10⁻³
30 20 10 5
I
II
III
RELATIVE A220nm
100
80
60
40
20
0
9
7
5
3
1
HYDROLYSIS TIME
(hr)
5 7 9 11 13 15 17
VOLUME (mℓ)

Figure 4. Gel filtration analysis of alkali-denatured (A) and acid-denatured glycinin (B) hydrolyzed by trypsin at pH 8.0 for 1.3 and 19 hours, respectively. The 220 nm absorbance is normalized relative to the highest peak of each sample for comparison.

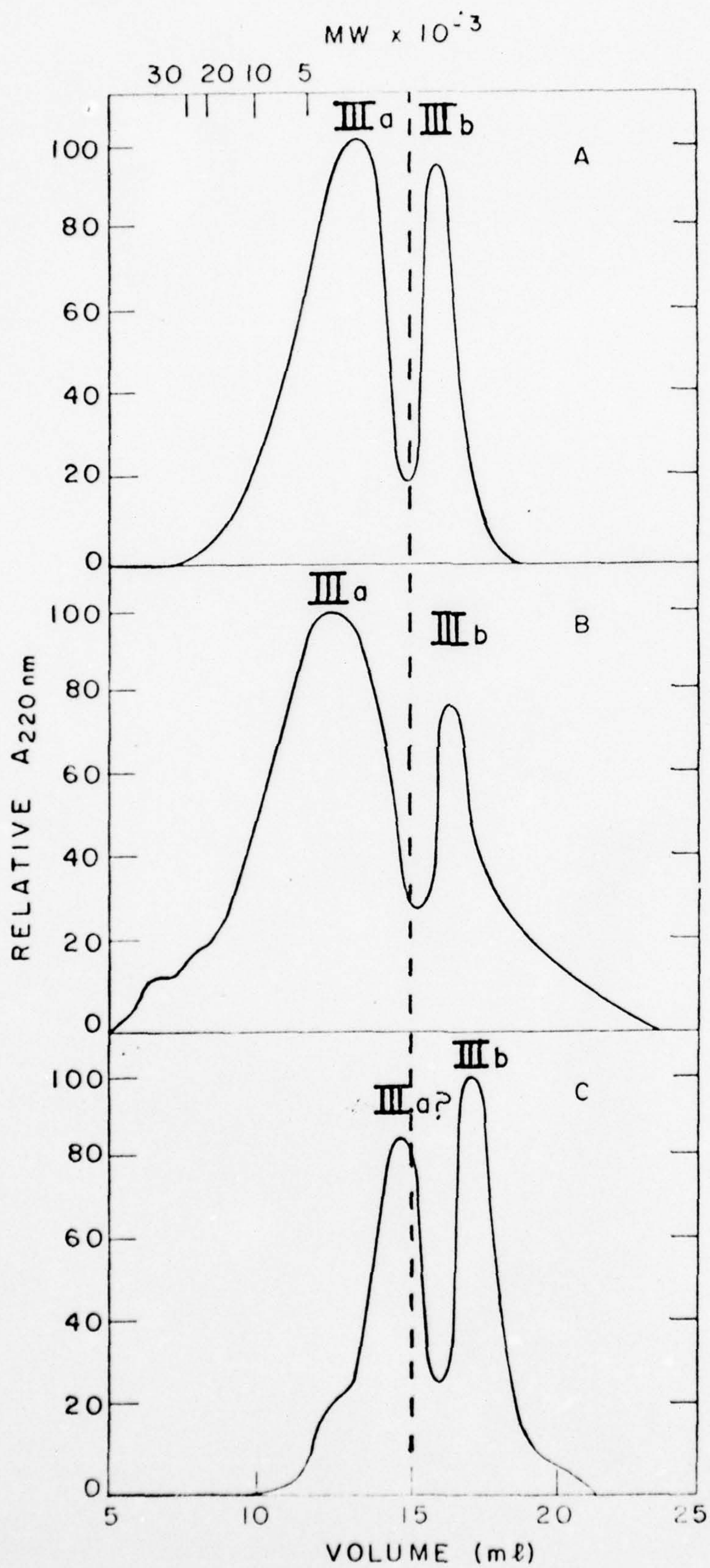

MW x 10^-3
30
20
10
5
100
80
60
40
20
0
A
B
I
II
IIIa
IIIb
RELATIVE A220 nm
5
10
15
20
25
VOLUME (ml)

are resolved in the low molecular weight range. Peak II is much smaller in the alkali-denatured protein and peak IIIb much larger than the corresponding peaks in the acid-denatured protein. In contrast to the above samples (Fig.5), CM-glycinin and its CM-acidic and CM-basic subunits upon completion of hydrolysis (i.e. 2.1, 1.4 and 16.5 hours, respectively) did not exhibit peaks I and II. Most of the hydrolyzed material was represented by peaks IIIa and IIIb. However, CM-glycinin and CM-acidic subunits produced significantly larger amounts of peptides in peak IIIa in comparison to the CM-basic subunits.

C. Conclusions from pH-Stat and Gel Filtration Data

Evidence has been provided that conformational changes in the structure of glycinin affect the rate and extent of hydrolysis of this protein by trypsin. Concerning the rate of hydrolysis as assessed by the pH-stat procedure, it appears that unfolding of glycinin in 6 M urea (17) followed by reduction of the SS bonds and blockage of the SH groups shortens dramatically the time required for completion of the hydrolysis in comparison to the native protein. A similar effect was observed by the action of alkali (preincubation at pH 12) which is known to unfold glycinin (17) and reduce

Figure 5. Gel filtration analysis of CM-glycinin (A), CM-acidic subunits (B) and CM-basic subunits (C) of glycinin hydrolyzed by trypsin at pH 8.0 for 2.1, 1.4 and 16.5 hours, respectively. The 220 nm absorbance is normalized relative to the highest peak of each sample for comparison.


MW x 10⁻³
30 20 10 5
IIIa
IIIb
A
B
IIIa?
C
RELATIVE A220nm
VOLUME (mℓ)
0
20
40
60
80
100
5
10
15
20
25

some susceptible SS bonds (M. Draper and N. Catsimpoolas, in preparation). Acid-induced denaturation (preincubation at pH 2.0) is less effective probably because unfolding of the protein does occur but the SS bonds are not cleaved, thus, providing a resistant core which is slowly attacked by the enzyme. A surprising finding was the observed differential rate of hydrolysis of unfolded. SS-reduced and SH blocked acidic and basic subunits of glycinin. The CM-acidic subunits exhibited the fastest rate of hydrolysis recorded in these experiments and CM-basic subunits the slowest. It is possible that the CM-basic subunits being more hydrophobic (8) than the acidic aggregate in such a fashion as to expose only a limited area to the enzyme. It should be mentioned that there is no considerable difference in the lysine and arginine content of these two types of subunits (8) to justify the obtained difference in rate. From the above data, the question may arise why is CM-glycinin digested so fast by trypsin, since, it contains CM-basic subunits. The only explanation we can offer at the present time is that the interaction of the acidic and basic subunits inhibits the aggregation of the basic subunits and, thus, the rate of hydrolysis remains high.

The molecular weight distribution of the glycinin peptides as determined by the 220 nm absorbance distri-

bution (12) Sephadex G-50 elutes, provides evidence that the native protein produces peptide fragments which can be broadly categorized as those above 30,000 daltons (I), those around 7,000 daltons (II) and below 5,000 daltons (III). It is possible that the 7,000 daltons peak represents peptides still held together by SS bonds. This is suggested by the fact that peak II is considerably reduced in the alkali-treated protein and almost absent in the samples of CM-glycinin, CM-acidic, and CM-basic subunits with a corresponding increase in the very low molecular weight peak IIIb.

Other differences in the molecular weight distibution that were noticed are as follows. The acidic subunits produced somewhat higher molecular weight peptides than the basic subunits. In all the denatured samples peak III of native glycinin was resolved into two subpeaks IIIa and IIIb. Explanation for these phenomena cannot be provided at present. In general, much higher resolution was obtained with the Sephadex column described in this paper than in previous work (13) employing microcolumn where all the peaks I, II and III were merged into one continuous distribution.

In conclusion, the most important finding of this study is that rate and extent of hydrolysis of glycinin

by trypsin is dramatically improved by denaturation of the protein and reduction of the disulfide bonds. If this holds true for the minor storage proteins of the soybean i.e. beta and gamma conglycinins, it would be economically advantageous to employ denaturation methods prior to enzymatic hydrolysis in industrial processes where hydrolyzed protein is produced.

D. SDS-PAGE Studies

The SDS-PAGE electrophoretic patterns of the hydrolysis of glycinin by pepsin at pH 2.0 as a function of time are shown in Figure 6. The results at both 25°C and 37°C clearly indicate that the protein is cleaved into fragments of lower molecular weight than either the heavy (H) or light (L) chains (6,11) of the protein within two minutes. The control glycinin solution incubated under identical conditions but without the enzyme exhibits no apparent change in its subunit composition. Since the hydrolysis proceeded at a very rapid rate at 25°C and 37°C, we intentionally slowed down the reaction by incubation at 4°C. This latter experiment enabled us to observe the differential rate of hydrolysis of the heavy and light chains of glycinin by pepsin. As it can be seen in Figure 6, the heavy (acidic) subunits are digested at a faster rate than the

Figure 6. SDS-PAGE patterns of the hydrolysis of glycinin by pepsin: (A) zero time; (B) 2 min; (C) 5 min; (D) 10 min; (E) 20 min; (F) 30 min; (G) 45 min; (H) 60 min; (I) 90 min; (J) zero time; (K) 2 min; (L) 4 min; (M) 6 min; (N) zero time; (O) 2 min; (P) 4 min; (Q) 6 min; (R) glycinin; (S to V) are the controls at zero time, 2 min, 4 min, and 6 min, respectively. Large H and L indicate the heavy and light subunits. The zero time aliquot was taken immediately after the addition of the enzyme to the protein solution.

4°C 25°C 37°C

H

L

+

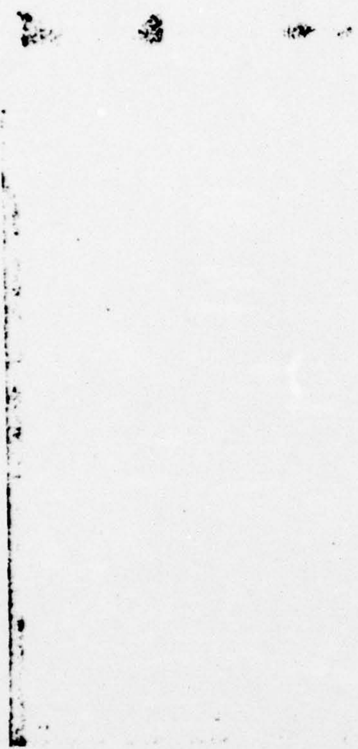
A B C D EFG H I J K L M NOPQ R S T U V

light (basic) subunits. In an analagous experiment using trypsin at pH 8.0 (25°C) we observed that although the rate of hydrolysis was at least ten-fold slower than the corresponding peptic digestion, the acidic subunits were again preferentially cleaved at a faster rate (Figure 7).

By using low molecular weight protein markers we have established the peptide fragments observed in Figure 1 are lower than 12,000 daltons and therefore fall outside the range of size estimated reliably by PAGE. However, by using Sephadex G-50 gel filtration (13), it has been demonstrated that the weight-average molecular weight of peptides produced by the action of pepsin on glycinin is in the range of 5,000 to 7,000 daltons.

The fast hydrolysis of glycinin by pepsin at pH 2.0 is probably due to the acidic dissociation of the subunits with concurrent unfolding of the polypeptide chain. Evidence for such conformational changes at pH 2.0 have been presented previously by hydrogen ion titration and uv difference spectroscopy of glycinin (17,18). The unfolded polypeptide chains are then presumably subjected to rapid proteolytic bonds. The synergistic effect of the acidic pH of pepsin activity aids to enhance the hydrolysis rate. One of the reasons for the observed slower hydrolysis rate by trypsin can probably be attributed to the fact that glycinin is conformationally stable at

Figure 7. SDS-PAGE patterns of the tryptic hydrolysates of glycinin at pH 8.0 at 25°C: (A)native glycinin; (B) zero time hydrolysate; (C) 2 min; (D) 30 min; (E) 90 min; (F) 150 min; (G) 240 min; (H) 300 min; (I) 24 hr; (J) standard proteins: albumin, ovalbumin, β-lactoglobulin, myoglobulin and lysozyme. The zero time aliquot was taken immediately after addition of the enzyme to the protein solution.



+
A B C D E F G H I J

pH 8.0. In addition, trypsin is restricted to the hydrolysis of lysine and arginine residues only.

The apparent preferential enzymatic hydrolysis of the acidic over basic subunits by both pepsin and trypsin is probably due to the greater hydrophobic character and therefore compactness of the basic subunits (8).

E. Ultraviolet Difference Spectra Studies

1. Preliminary considerations

The ultraviolet difference spectra of glycinin, the major storage protein of soybean seeds, (*Glycine max*) have been investigated previously by Fukushima (9), Catsimpoolas *et al*. (17), and Koshiyama and Fukushima (19). It has been shown that conformational changes (unfolding) induced by urea, guanidine hydrochloride, and acidic or alkaline pH result in the exposure of buried tyrosine and tryptophan groups which can be measured spectrophotometrically. Of particular interest was to determine if conformational changes occur gradually during hydrolysis by trypsin - at a pH in which the protein is stable in the absence of the enzyme - and if these changes can be detected by ultraviolet difference spectra. In the present study, we report that the method can be used to follow

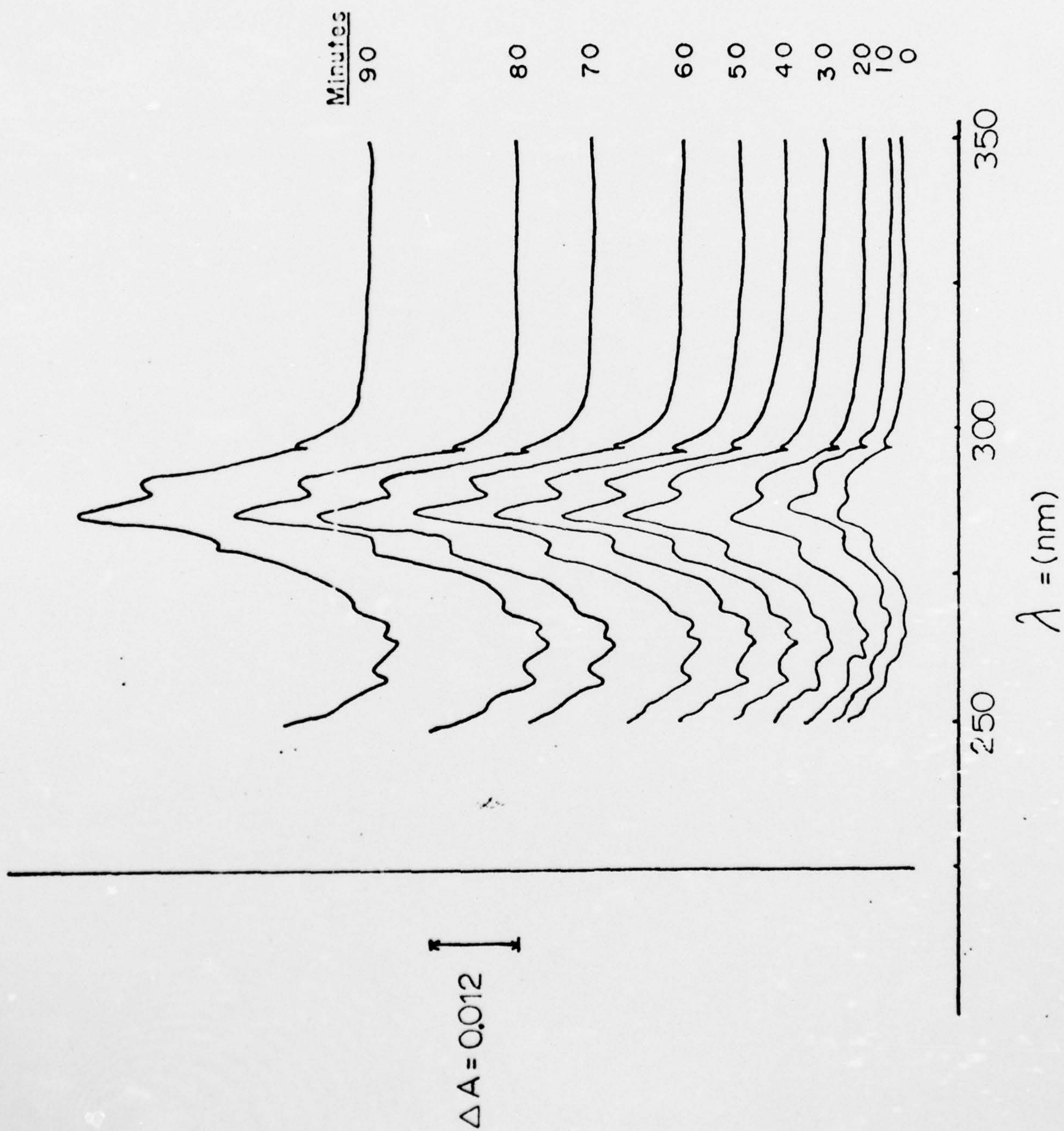
the exposure of buried chromophoric groups during enzymatic digestion of glycinin and to obtain relative rates of exposure as a function of ionic strength.

2. Findings and conclusions

Typical ultraviolet difference spectra of glycinin subjected to tryptic hydrolysis at pH 8.0 as a function of time are shown in Figure 8. The characteristic peak at 291-292nm originating largely from the exposure of tryptophan groups and the tyrosine-tryptophan peak at 286-287nm are clearly distinguished. Increasing exposure of both tyrosine and tryptophan groups were observed with increasing hydrolysis time producing the characteristic "denaturation blue shift" in the spectrum of glycinin (17). The small difference spectrum seen at "zero" time represents a few seconds of reaction during the mixing of the enzyme with the protein.

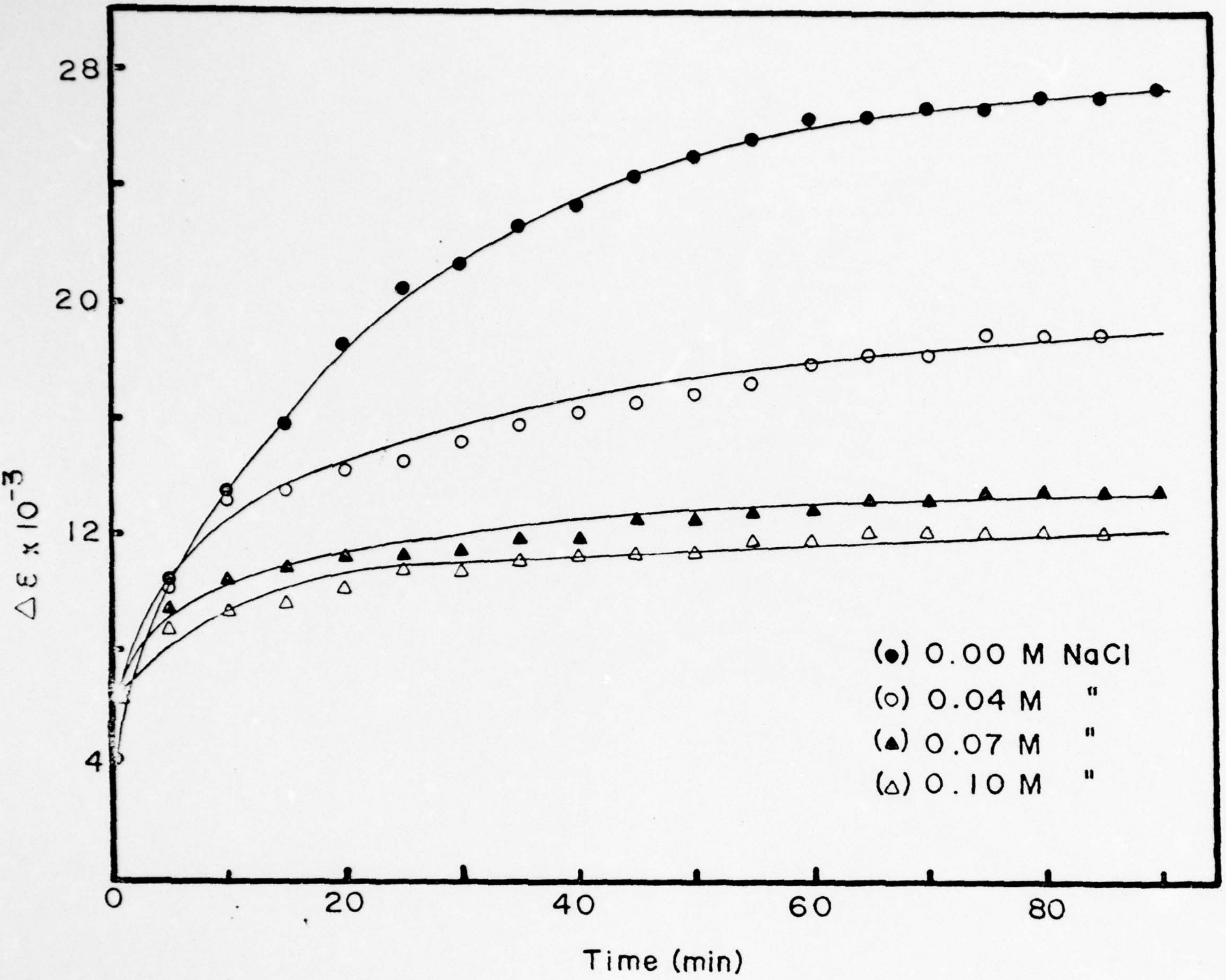
Since the spectrum at 291-292nm represents the exposure mainly of only one type of chromophoric groups (i.e. tryptophan), it was chosen for the kinetic studies in order to avoid complications due to the simultaneous exposure of two types of groups (286-287nm spectrum). The time course of the molar absorptivity difference ($\Delta\varepsilon$) at 291-292nm and in the presence of various salt (NaCl) concentrations is shown in Figure 9. The extent

Figure 8. Ultraviolet difference spectra of glycinin induced by tryptic hydrolysis (pH 8.0) as a function of time.



Minutes
90
80
70
60
50
40
30
20
10
0
250
300
350
λ = (nm)
ΔA = 0.012

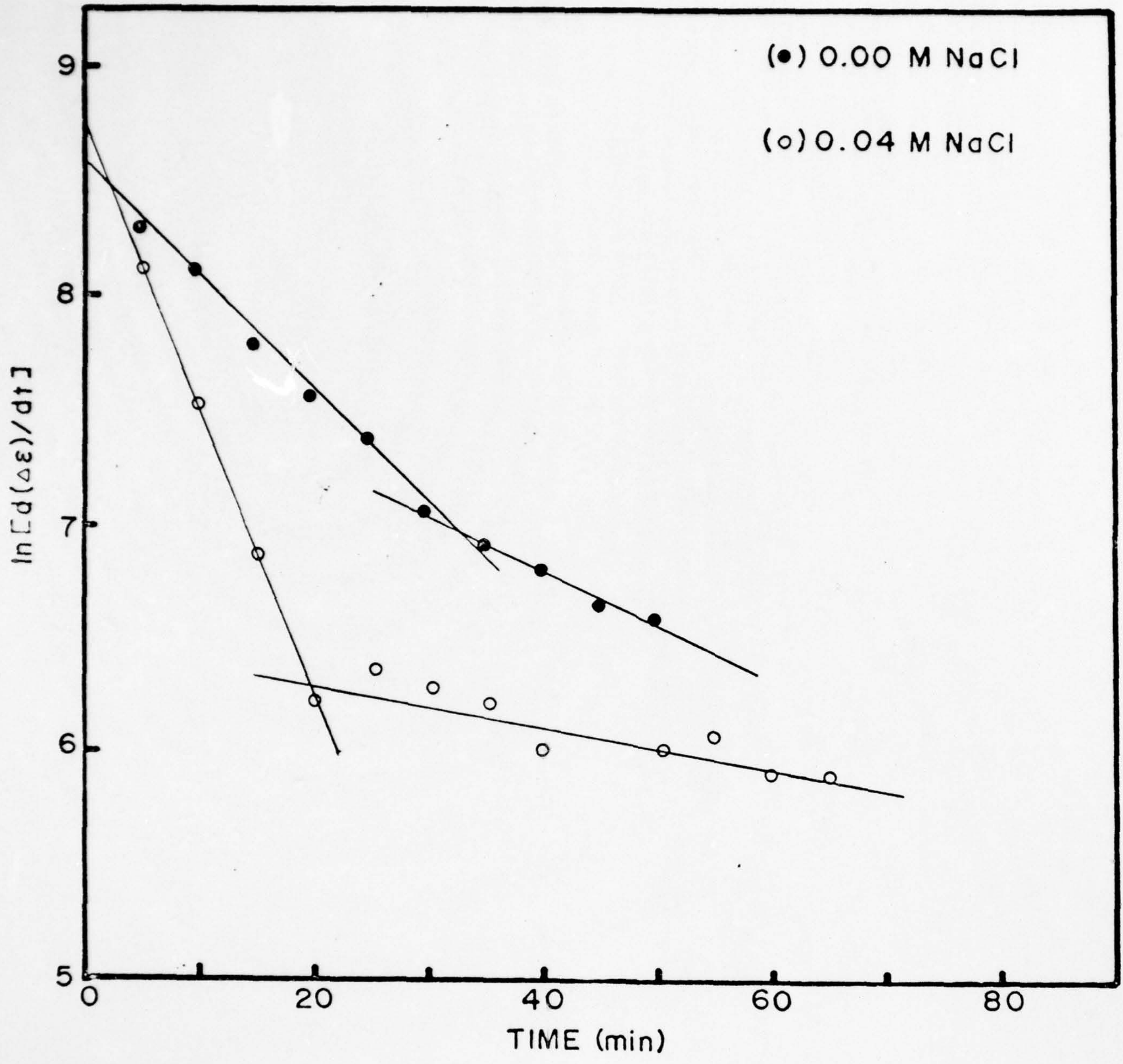
Figure 9. Time course of molar absorptivity differences ($\Delta\varepsilon$) at 291-292nm obtained in the presence of various concentrations of NaCl during tryptic hydrolysis of glycinin.


28
20
12
4
Δε x 10^-3
0
20
40
60
80
Time (min)
(●) 0.00 M NaCl
(○) 0.04 M "
(▲) 0.07 M "
(△) 0.10 M "

of exposure was diminished by the increasing ionic strength of the incubation mixture. Even in the absence of NaCl, the exposure of tryptophan groups in hydrolyzed glycinin amounted to only approximately 75% of the exposure estimated previously (17) in the presence of 6 M urea. Thus, it appears that after tryptic hydrolysis, the peptide fragments of glycinin retain some residual structure capable of burying tryptophan groups. This finding is not in conflict with molecular weight distribution studies of tryptic digests of glycinin where the existence of high molecular weight peptides (e.g. >6,000) has been demonstrated. Such large fragments may maintain some of their internal structure.

In an attempt to estimate the rate of change in $\Delta\varepsilon$ as an index of exposure of buried groups, it was observed that at all ionic strengths tested, two types of kinetics existed. A fast (20 to 30 minutes) and a subsequent slow exposure of tryptophan groups took place as illustrated in Figure 10. Plots of the fast (K_f) and slow (K_s) rate constants at 291-292nm as a function of NaCl concentration are depicted in Figure 11. It may be seen that the effect of ionic strength is much more pronounced on K_f than on K_s.

Figure 10. Typical plot to estimate the fast and slow exposure rates at 291-292nm.


(●) 0.00 M NaCl
(○) 0.04 M NaCl
ln[d(Δε)/dt]
9
8
7
6
5
0
20
40
60
80
TIME (min)

AD-A042 461 MASSACHUSETTS INST OF TECH CAMBRIDGE DEPT OF NUTRITI--ETC F/G 6/1
SULFHYDRYL AND DISULFIDE GROUPS IN GLYCININ. THE MAJOR SOYBEAN --ETC(U)
1977 N CATSIMPOOLAS DAHC04-74-C-0029

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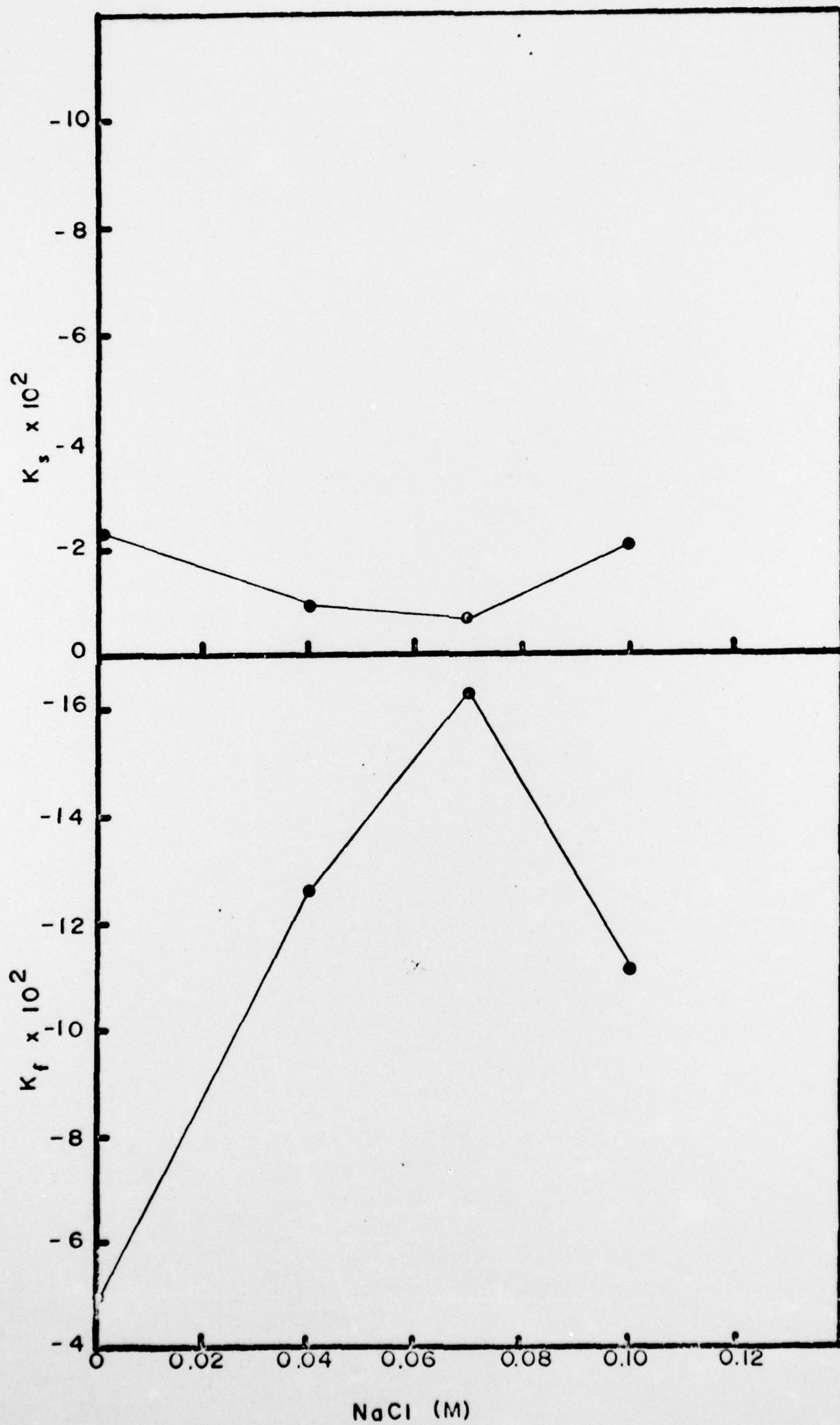
Figure 11. Plot of fast (K_f) and slow (K_s) rate constants as a function of NaCl concentration.


-10
-8
-6
-4
-2
0
$K_s \times 10^2$
-16
-14
-12
-10
-8
-6
-4
$K_f \times 10^2$
0
0.02
0.04
0.06
0.08
0.10
0.12
NaCl (M)

The presence of two exposure rates may reflect the differential hydrolysis of the acidic and basic subunits of glycinin where ionic strength effects may play a more important role in the stabilization of their internal structure of the acidic subunits than the basic ones.

Indeed, experiments described above using isolated acidic and basic subunits of glycinin (11) have also provided evidence for the faster digestion of the acidic subunits in comparison to the basic ones.

Since, the demonstration of the existence of acidic and basic subunits in glycinin (4), we have been wondering what the physiological importance of this finding may be. We are now proposing that the two types of subunits act as regulators of their own hydrolysis in the germinating seed in conjunction with the local concentration of salts. The acidic subunits may provide needed peptide fragments at the initial stages of germination whereas the basic subunits are involved in a longer term supply of amino acids. The salt concentration at a particular stage of germination may speed up or slow down the enzymatic hydrolysis. Since the type of proteolytic enzymes present in soybeans is unknown, trypsin may be as good a model of action as any other enzyme operating out a non-acidic pH.

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VI. Publications (Supported by Contract)

N. Catsimpoolas

Immunological properties of soybean proteins. In: Immunological Aspects of Foods (N. Catsimpoolas, ed.) AVI Publishing Co., Westport, Conn. (1976) pp. 37-59

M. Draper and N. Catsimpoolas

Isolation of the acidic and basic subunits of glycinin

Phytochemistry 16: 25-27

M. Draper and N. Catsimpoolas

Disulfide and Sulfhydryl Groups in Glycinin

Cereal Chem. (submitted)

C.J. Lynch, C. Rha, and N. Catsimpoolas

Tryptic hydrolysis of glycinin and its subunits

J. Sci. Fd. Agric. (submitted) (partial support)

C.J. Lynch, C. Rha, and N. Catsimpoolas

Note on the Rapid Proteolysis of Glycinin by Pepsin and Trypsin

Cereal Chem. (in press) (partial support)

Z. Ardalan - deWeck, C. Rha and N. Catsimpoolas

Ultraviolet difference spectra of glycinin induced by tryptic hydrolysis

Cereal Chem. (submitted) (partial support)

VII. Personnel Supported

1. Nicholas Catsimpoolas, Ph.D.
   Associate Professor

2. Marta Draper, B.S. (Ph.D. June 1977)
   Research Assistant

3. Ann L. Griffith, Ph.D.
   Research Associate

4. Pat Kien, B.S.
   Technical Assistant

A Ph.D. degree in Nutritional Biochemistry and Metabolism was awarded (June 1977) to Marta Draper, her thesis entitled "Structural Studies of Glycinin: the Major Storage Protein of the Soybean" was supported entirely by the present contract.