Pergamon

0031-9422(95)00896-9

Phytochemistry, Vol. 42, No. 2, pp. 313–319, 1996

0031-9422/96 $15.00 + 0.00

AN ACIDIC AMINO ACID-SPECIFIC PROTEASE FROM GERMINATING SOYBEANS

ANNA L. TAN-WILSON, XIAOWEN LIU, RUOYING CHEN,* XIAOQUN QI and KARL A. WILSON

Department of Biological Sciences, State University of New York at Binghamton, Binghamton, NY 13902-6000, U.S.A.

(*Received in revised form* 31 *October* 1995)

Key Word Index—Acidic amino acid-specific protease; glutamic acid-specific protease; aspartic acid-specific protease; serine protease; soybeans; mung bean; *Vigna radiata*; *Glycine max*; Leguminosae; cotyledons; germination; β-conglycinin; storage protein degradation.

Abstract—The degradation of the β-conglycinin protein reserves in soybean seeds during germination and early growth begins with the proteolysis of its α and α′ subunits by an enzyme called Protease C1. In the pathway, a number of proteolytic intermediates are produced and subsequently degraded. Determination of the N-terminal sequences of these intermediates provides insight regarding the requirements of the cleavage sites. The N-terminal sequence of three such proteolytic intermediates has been determined. The sequence has been located in the published sequences of the β-conglycinin subunits. Comparing these cleavage sites, plus those of two others previously delineated, shows that the P1′ and P4′ positions always bear either a Glu or an Asp residue while the P1 position always bears either a Glu or a Gln residue. In addition, other sites from P3 to P7′ are also rich in either Glu or Asp, and the whole region is predicted to be in an α-helix. Consistent with the observation, synthetic poly-L-Glu inhibits the Protease C1-catalysed degradation of the α and α′ subunits of β-conglycinin. Poly-L-Glu (av. $M_r = 1000$) at 12.5 mM was more effective at inhibiting the reaction than poly-L-Glu (av. $M_r = 600$) or poly-L-Glu (av. $M_r = 14\,300$) at the same concentration. Comparing large synthetic polypeptides at 12.5 mM, inhibition by poly-L-Asp (av. $M_r = 15\,000$) is as effective as poly-L-Glu (av. $M_r = 14\,300$), while poly-L-Ser (av. $M_r = 15\,000$) had no effect at all. Poly-D-Glu (av. $M_r = 15\,000$) is a better inhibitor than poly-L-Glu of the same size. A serine protease of similar molecular weight as Protease C1 and also capable of catalysing the proteolysis of the α and α′ subunits of β-conglycinin to generate proteolytic intermediates of the same size has been found in mung bean.

INTRODUCTION

Proteolysis of seed storage proteins is a critical step in the process of germination and early growth. In the early stages, native protein subunits are cleaved at only a few sites, generating proteolytic intermediates that are then susceptible to a host of more general proteolytic enzymes [1]. Enzymes that recognize the native protein subunits and catalyse limited proteolysis have been found to have narrow specificity for the amino acid residues contributing to the peptide bond to be cleaved. One such enzyme that participates in the initial degradation pathway in germinating vetch has been shown to cleave specifically peptide bonds that have an Asn contributing the carboxylic acid group. This enzyme also functions as a protein processing enzyme in developing seeds and belongs to a family of other vacuolar protein processing enzymes including the enzymes found in soybean [2], castor bean [3, 4], jack bean [5] and pumpkin [6]. Asparaginyl-specific proteases have also been found in germinating garden bean [7] and moth bean [8].

In soybean seedling cotyledons, proteolysis of the storage proteins is also initiated by proteolytic enzymes with narrow substrate specificity. For example, the Kunitz trypsin inhibitor that is found in protein bodies in soybean cotyledons loses five C-terminal residues—DKESL—through cleavage of a Leu–Asp bond [9]; and a Bowman–Birk trypsin inhibitor loses a C-terminal dipeptide—EN—through cleavage of a Lys–Glu bond [10]. Cysteine proteases are involved [11, 12] and the proteolytic intermediate is very quickly degraded by other enzymes of wider specificity so that no further discrete intermediates are detected.

The degradation of the major storage proteins in the soybean also begins with limited proteolysis during early growth. The β-conglycinins constitute approximately half of the major storage proteins in the soybean. These proteins are trimers of three subunits: α′, α and β, appearing in all possible combinations

*Present address: Pioneer Hi-bred International, Inc., Plant Breeding Division, Johnston, IA 50131-0038, U.S.A.

[13]. There is a 93% amino acid sequence identity between the α' and α subunits. The β subunit is missing a segment of 179 amino acid residues at the N-terminus of the α' and α subunits; and shows 75% sequence identity to the remainder of those two subunits [14]. An enzyme that initiates the degradation of β-conglycinin, one of the two major storage proteins of soybean, does so by cleaving only its α' and α subunits, leaving the β subunit intact. The first two proteolytic intermediates generated have molecular masses that are only 1–2 kD lower than those of the native subunits. Subsequently, these two intermediates are cleaved and a series of smaller proteolytic intermediates appear until the final enzymic products of molecular masses, 50 and 48 kD, are produced [15]. The same proteolytic intermediates are seen in the cotyledons *in vivo* [16]. The 50 and 48 kD products are relatively stable intermediates which are eventually degraded to smaller polypeptides, presumably by other enzymes [15].

We have previously reported the purification and characterization of this enzyme, which we called Protease C1, because it is the first proteolytic enzyme attacking β-conglycinin [17]. Protease C1 is a serine protease and works at acidic pH. In addition to catalysing proteolysis of the soybean β-conglycinin, it also cleaves structurally homologous storage proteins such as convicilin from pea, and the acidic subunits of the other major set of storage proteins in the soybean, the glycinins. Comparison of the amino acid sequences of proteins that served as substrates and those that do not led us to hypothesize that Protease C1 cleaves wherever an exposed portion of a polypeptide chain bears a string of acidic amino acid residues. Indeed, isolation of two proteolytic intermediates and determination of their amino-terminal sequences showed that cleavage occurred between two acidic amino acid residues, in a region of the polypeptide that bears many other acidic amino acid residues [17].

The information gleaned from only two cleavage sites is insufficient for us to determine the minimum requirements for a cleavage site for Protease C1. We have now purified three more of the proteolytic intermediates and determined the sequences at the cleavage sites. We also present results of inhibition studies using polymers of acidic amino acids and report on a similar enzyme in the cotyledons of mung bean seedlings.

RESULTS

Amino acid sequence of the cleavage site

Purified β-conglycinin from soybean was incubated with pure protease C1. The electrophoresis gels showed that only the α and α' subunits of β-conglycinin are cleaved, generating a series of proteolytic intermediates. Using the procedure described in the Experimental section, we succeeded in obtaining the 50, 58 and 65 kD bands for N-terminal sequence analyses. The single N-terminal amino acid sequence obtained for each band showed that this procedure for generating the intermediates was sufficient to produce pure intermediates.

The N-terminal sequences of the three intermediates were as follows:

50 kD	EEEDQDEDEE-
58 kD	DEREHPRPHQ-
65 kD	EEEEGEIPRP-

The results show that the first two and the fourth amino acids for the three intermediates were acidic amino acids, either Glu or Asp. Both the 50 and 65 kD intermediates also had an overall preponderance of acidic amino acid residues in the 10-amino acid N-terminal sequence.

The origins of these proteolytic intermediates can be determined since nucleotide sequences of the cDNAs corresponding to the α and α' subunits of β-conglycinin, and their derived amino acid sequences, have been reported [18]. By comparing the N-terminal amino acid sequences of the proteolytic intermediates to the published sequences [18], we were able to pinpoint the cleavage sites on the β-conglycinin that gave rise to these intermediates. The N-terminal amino acid residues of the 50 and 58 kD intermediates were found to be amino acid residues 178 and 143 of the α' subunit, respectively. The N-terminal amino acid of the 65 kD intermediate was found to be residue number 67 of the α subunit. The positions of the α' and α subunit are counted from the N-terminal amino acid of the precursor proteins. For both subunits, the N-terminal amino acid of the mature protein is the 63rd residue of the precursor [18]. There were no other sites on the α', α or β subunits that would have accounted for the N-terminal sequences of the intermediates. The molecular masses of each of the isolated intermediates corresponded to the expected molecular masses of the C-terminal portion of the α or α' subunits if cleaved at the delineated cleavage sites. The amino acid sequences around the cleavage sites that generated the three intermediates are shown in Fig. 1. Two other cleavage sites that had been determined previously [17] are also shown in the figure. Using the nomenclature introduced by Schecter and Berger [19], the amino acid residues at the P1 and P1′ sites, at which cleavage occurs, are either glutamate, asparate or glutamine. Several adjacent acidic amino acid residues flank these two amino acid residues.

The secondary structures at these cleavage sites predicted by the principles formulated by Chou and Fasman [20] are also shown in Fig. 1. The amino acid sequences around the three cleavage sites are predicted to be in α-helical regions as shown in Fig. 1. To generate the 50 and 58 kD polypeptides, the amino acid contributing the carboxyl group of the peptide bond cleaved is four amino acid residues away from the amino-terminal boundary of α-helical regions. To generate the 65 kD polypeptide, cleavage is 15 residues away from the N-terminal boundary of an α-helical region.

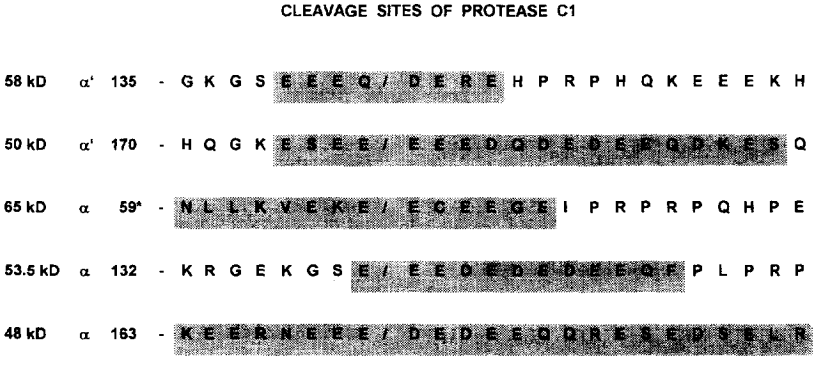

Fig. 1. Amino acid sequences at sites where soybean β-conglycinin is cleaved by soybean Protease C1 to generate the 50 kD product and the 58 and 65 kD proteolytic intermediates. The sequences are form Sebastiani *et al.* [18]. Numbers are of the leftmost amino acid residue listed, counting from the amino-terminus of the precursors of the α′ or α subunits of β-conglycinin. Shaded residues are in predicted α-helical regions according to the rules of Chou and Fasman [20]; and the slash (/) indicates the peptide bond cleaved.

Inhibition of Protease C1 by poly-L-Glu

Glutamic acid or synthetic polyglutamic acid polypeptides might be expected to inhibit the cleavage of β-conglycinin by Protease C1 if the presence of acidic amino acid residues were indeed important to the cleavage site. To test this hypothesis, the effect of adding glutamic acid and poly-L-glutamic acid to the standard reaction mix was determined. The results in Fig. 2 show that glutamic acid did not cause appreciable inhibition of Protease C1 activity, while poly-L-Glu of average M_r 1000 did. Activity measured as the disappearance of the α′ subunit of β-conglycinin in the absence of glutamic acid or poly-L-Glu was 7.12 μg of α′ subunit cleaved per hour during the first 2.5 hr, under the conditions of this assay. In the presence of poly-L-Glu (average M_r 1000), activity was 0.23 μg of α′ subunit disappeared per hour, equivalent to 96.8% inhibition.

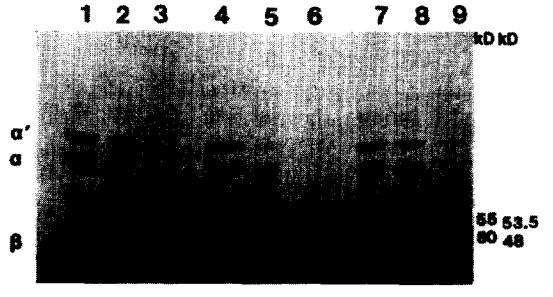

Fig. 2. SDS-PAGE showing the effect of the addition of 50 mM glutamic acid and 50 mM poly-L-Glu (average M_r 1000) to the reaction of Protease C1 with soybean β-conglycinin. Lanes 1, 2 and 3 show the reaction without any additions at 0, 2.5 and 24 hr, respectively. Lanes 4, 5 and 6 show the reaction with added glutamic acid at 0, 2.5 and 24 hr, respectively. Lanes 7, 8 and 9 show the reaction with added poly-L-Glu at 0, 2.5 and 24 hr, respectively. The bands corresponding to the α′, α and β subunits of β-conglycinin are marked, as are some of the proteolytic intermediates.

Upon 24-hr incubation, when the α′ and α subunits of β-conglycinin have all been cleaved, inhibition is still evident when one examines the proteolytic intermediates and products. The 50 and 48 kD products are clearly distinct in the control reaction, but can barely be discerned in the reaction mix with poly-L-Glu (average M_r 1000). Instead, the 55 and 53.5 kD intermediates on the pathway to the 50 and 48 kD products are still very prominent. The 24-hr reaction mix in the presence of glutamic acid shows some slowing down of the process of degradation, but not to the same extent as seen with the polypeptide.

Later, we obtained a shorter poly-L-Glu (average M_r 600). Comparing its effect with that of poly-L-Glu (average M_r 1000) at 12.5 mM, with activity measured as the disappearance of the α′ subunit of soybean β-conglycinin, inhibition by poly-L-Glu (average M_r 600) was only 30%, while inhibition of digestion by the longer Glu (average M_r 1000) polypeptide was close to 100%.

Inhibition of Protease C1 by other amino acid polymers

Considering the presence of multiple glutamic and aspartic acid residues at the cleavage sites, we would expect poly-L-Asp to show inhibition of Protease C activity. We tested this, and also tested poly-L-Ser as a homopolymer of an amino acid that is not featured strongly at the cleavage site for this enzyme. Finally, we tested the effectiveness of poly-D-Glu as an inhibitor. The polypeptides used were polymers of average M_r from 14 300 to 15 000 since these were commercially available, whereas shorter polypeptides were not. Figure 3 shows that the proteolysis of soybean β-conglycinin by Protease C1 is still inhibited by the

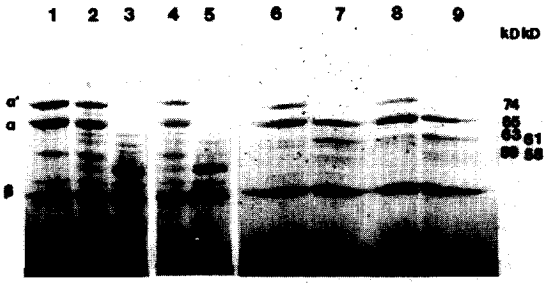

Fig. 3. SDS-PAGE showing the effect of addition of 12.5 mM poly-L-Ser (average M_r 15 000), poly-L-Glu (average M_r 14 300) and poly-L-Asp (average M_r 15 000) to the reaction of Protease C1 with soybean β-conglycinin. Lanes 1, 2 and 3 show the reaction without any additions at 0, 2.5 and 24 hr, respectively. Lanes 4 and 5 show the reaction with added poly-L-Ser at 2.5 and 24 hr, respectively. Lanes 6 and 7 show the reaction with poly-L-Glu at 2.5 and 24 hr, respectively. Lanes 8 and 9 show the reaction with poly-L-Asp at 2.5 and 24 hr, respectively. The bands corresponding to the α', α and β subunits of β-conglycinin are marked, as are some of the proteolytic intermediates.

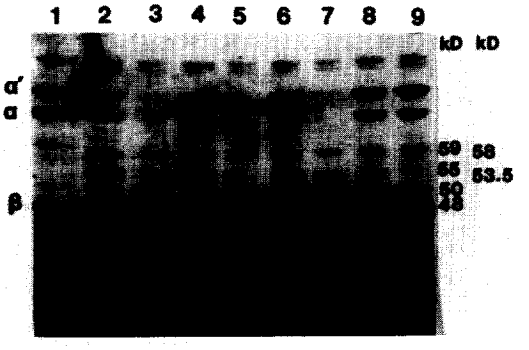

Fig. 4. SDS-PAGE showing the effect of addition of 75 μM poly-L-Glu (average M_r 1000), poly-L-Glu (average M_r 14 300) and poly-D-Glu (average M_r 15 000). Lanes 1, 2 and 3 show the reaction without any additions at 0, 2.5 and 24 hr, respectively. Lanes 4 and 5 show the reaction with added poly-L-Glu (average M_r 1000) at 2.5 and 24 hr, respectively. Lanes 6 and 7 show the reaction with added poly-L-Glu (average M_r 15 000) at 2.5 and 24 hr, respectively. Lanes 8 and 9 show the reaction with added poly-D-Glu (average M_r 15 000) at 2.5 and 24 hr, respectively. The bands corresponding to the α', α and β subunits of β-conglycinin are marked, as are some of the proteolytic intermediates.

poly-L-Glu of average M_r of 14 300, although not to the same extent obtained with the shorter poly-L-Glu (Fig. 2). When the reaction was conducted in the presence of 12.5 mM poly-L-Glu (average M_r 14 300), the enzyme activity measured as the disappearance of the α' subunit of β-conglycinin at 2.5 hr was lower than that of the uninhibited reaction by 11%. The larger polypeptide may be inhibited from approaching the enzyme's active site by its folded structure. Nevertheless, it still worked as an inhibitor of Protease C1. Thus, we proceeded to compare the extent of Protease C1 inhibition achieved in the presence of poly-L-Glu, poly-L-Asp, poly-L-Ser and poly-D-Glu of similar sizes.

Figure 3 shows that the additional of poly-L-Asp (average M_r of 15 000) at 12.5 mM also resulted in 11% inhibition of Protease C1 activity when measured within the first 2.5 hr. By 24 hr, when the α' and α subunits have all been cleaved even in the presence of poly-L-Glu of poly-L-Asp, inhibition is indicated by the continuing presence of the 65 and 63.5 kD proteolytic intermediates, intermediates that are degraded by this time in the uninhibited reaction. Poly-L-Ser (average M_r 15 000) at 12.5 mM did not inhibit the reaction. The α' and α subunits of β-conglycinin were degraded, the proteolytic intermediates appeared and the 50 and 48 kD final products were made as in the uninhibited reaction, indicating that not just any polypeptide inhibits Protease C1 (Fig. 3). Thus, this experiment shows that both poly-L-Glu and Poly-L-Asp at concentrations of 12.5 mM exert about the same inhibitory effect on the activity of Protease C1; while poly-L-Ser, not bearing acidic amino acid residues, does not inhibit the enzyme.

To examine the effect of poly-D-Glu (average M_r 15 000), we had to use concentrations of 75 μM, the limit of solubility for the poly-D-Glu in our reaction mix. We compared its effect to that of poly-L-Glu (average M_r 14 300). The results are shown in Fig. 4. At this low concentration of poly-L-Glu (average M_r 14 300), the activity of the uninhibited control reaction was 6.08 μg of α' subunit degraded per hour. The addition of 12.5 mM poly-L-Glu (average M_r 14 300) or of poly-L-Glu (average M_r 1000) showed no inhibition at this concentration. Poly-D-Glu (average M_r 15 000), on the other hand, exerted almost 100% inhibition, showing itself to be by far the best inhibitor of those tested.

Homologous enzyme in another legume species

Soybean Protease C1 has characteristics very similar to that of another serine proteolytic enzyme in mung bean, Protease F [21, 22]. The mung bean enzyme is also a serine protease with a pH optimum of 4.0. It has an M_r of 65 000, similar to the M_r of 70 000 or Protease C1. The mung bean enzyme is found in protein bodies and is active during early growth. It is called Protease F because it cleaves MBTI-F, the main form of a Bowman–Birk-like trypsin inhibitor in mung bean seeds. Protease F cleaves the Asp–Lys peptide bond in the acidic amino acid-rich -DKDDD sequence at the carboxyl-terminus of mung bean trypsin inhibitor F [21, 22]. This similarity prompted us to test pure mung bean Protease F with soybean β-conglycinin as a substrate. Soybean β-conglycinin was incubated with Protease F at pH 4.5 for 24 hr using assay conditions set up originally for soybean Protease C1 [16]. Figure 5 shows that the α' and α subunits of β-conglycinin were digested by mung bean Protease F. As with Protease C1 of soybean, both the α' and α subunits disappear, leaving the β subunit intact. Like Protease C1, digestion of the α subunit (61% disappearance) is more

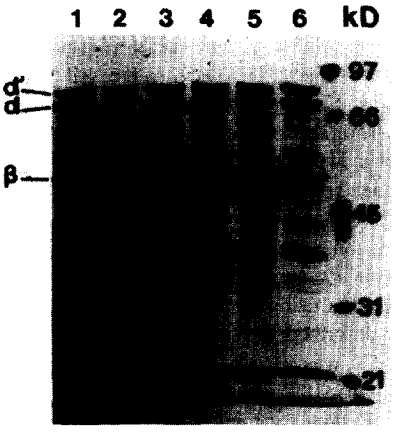

Fig. 5. SDS-PAGE showing mung bean Protease F activity with soybean β-conglycinin as substrate. β-Conglycinin incubated with Protease C1 for 0 hr (lane 1) and 2 hr (lane 2) or β-conglycinin incubated alone for 2 hr (lane 3). β-Conglycinin incubated with Protease F for 0 hr (lane 4) and 2 hr (lane 5) or β-conglycinin incubated alone for 2 hr (lane 6). Positions of the α′, α and β subunits of β-conglycinin as well as molecular masses of standard indicators are shown.

extensive than digestion of the α′ subunit (33% disappearance). The 63 and 61 kD proteolytic intermediates that we saw with soybean Protease C1 digestion also appeared. The smaller proteolytic intermediates and final products (50 and 48 kD) that we normally see upon incubation of Protease C1 with soybean β-conglycinin did not, however, appear during the 24 hr of incubation of the reaction mix.

DISCUSSION

The cleavage specificity of Protease C1 for peptide bonds involving acidic amino acid residues has been shown for a few other proteases. Proteolytic enzymes that cleave on the carboxyl side of either Glu or Asp residues have been found in bacteria [23–26]. Unlike Protease C1, bacterial enzymes accept a large number of different amino acids at the P1′ position of the cleaved peptide bond. Even more unlike Protease C1, polypeptides that have acidic amino acid residues at the P2 site are poor substrates for the bacterial enzymes [27]. Furthermore, these enzymes work optimally at pH values ranging from 7.5 to 8.8; while soybean Protease C1 works optimally at pH 4.5. Protease C1 is also different from the asparaginyl-specific vacuolar processing enzymes found in plants [2–8] in that none of the cleavage sites delineated for Protease C1 so far have an Asn at the P1 site.

Other serine proteases have been purified from soybean, e.g. the glycinin A_4A_5 digesting protease that works best at pH 8.5 [28] and a protease that acts specifically on the α but not the β subunit of β-conglycinin. The latter enzyme works at pH 8 and cleaves between two arginine residues [29]. These are clearly distinct from Protease C1 in pH optimum and cleavage specificity. A plant proteolytic enzyme that soybean Protease C1 more closely resembles in cleavage specificity is one from germinating sorghum that recognizes peptide bonds in which the amino acid residue contributing the carboxyl group is a Glu or an Asp. The sorghum enzyme, however, was an aspartic, not a serine protease [30].

In this report, we have delineated three sites cleaved by Protease C1. We had previously located two other sites [16]. When comparing the five sites (Fig. 6), the amino acid residues at P1′ and P4′ are always either a Glu or an Asp. If this were the only criterion for the Protease C1 cleavage site, then there would be other cleavage reactions in addition to these five sites and we would not be able to obtain discrete amino-terminal sequences. We thus hypothesize that Protease C1 binding to substrate may also involve Gln residues. Although Glu and Asp are often thought to be ionized at the acidic pH conditions that we use in our assay, in fact, the proximity of many negatively charged amino acid residues would reduce the degree of ionization. Thus there may not be a charge difference between some Gln and Glu residues in these sequences.

Re-examining the five cleavage sites that we have delineated so far, we hypothesize that positions P1′ and P4′ always bear a Glu or an Asp, while position P1 always bears either a Glu or a Gln. A requirement for multiple binding sites on the enzyme could explain why proteolysis is limited and results in discrete intermediates rather than a large set of intermediates that would be seen as a smear on SDS-PAGE.

The amino acids at positions outside of P1, P1′ and P4′ may also be important in substrate binding. Four of the five cleavage sites delineated have either Glu, Asp or Gln at positions P3, P2′, P3′ and P6′. Three of the five sites bear Glu, Asp or Gln at positions P2, P5′ and P7′. We have found that all cleavage sites are in regions

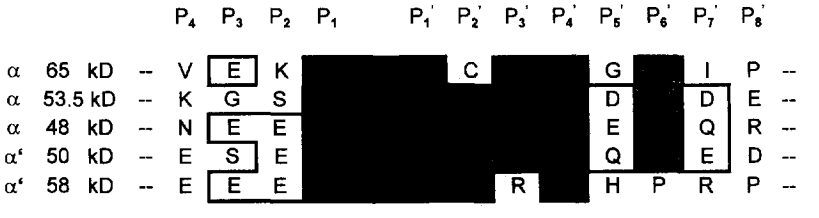

Fig. 6. Comparison of the amino acid sequences at five cleavage sites recognized by soybean Protease C1. The peptide bond cleaved is between the amino acid residues at P_1 and P_1'. Areas are boxed and shaded differently to designate the positions at which five, four and three amino acid residues are either Glu (E), Asp (D) or Gln (Q).

predicted to be α-helical. We do not know whether positions outside of P1, P1′ and P4′ specifically need to be Glu, Asp or Gln for optimum cleavage susceptibility, or whether any residue allowing the formation of the α-helix is all that is required. We note the coincidence that cleavage at all but one position is one, two or four complete turns of the predicted helix from either the amino-terminal or the carboxyl-terminal boundary of the predicted α-helical region. One can also reasonably assume that regions so rich in the polar and sometimes charged amino acid residues, Glu, Asp and Gln, would be on an outer, accessible surface of the protein substrate.

Inhibition by polymers of L-glutamic and L-aspartic acids, but not by a polymer of serine, supports our hypothesis with regard to the importance of Glu and Asp in the substrate cleavage site. We also observed that poly-L-Glu (average M_r 1000) with about seven or eight glutamic acid residues is a better inhibitor compared to poly-L-Glu (average M_r 600) with about four or five glutamic acid residues. This observation supports our suggestion that the substrate binding site on the enzyme calls for binding of at least three specific amino acid residues that are five residues apart on the polypeptide chain. We reason that a polypeptide chain made up entirely of glutamic acid residues could bind in many ways to the substrate binding site on the enzyme. The probability that the longer poly-L-Glu would bind at the three critical sites five residues apart would be greater than for the shorter polypeptide, thus making the slightly longer polypeptide a better inhibitor in the degradation of the true substrate, β-conglycinin.

When comparing the larger polypeptides of average M_r *ca* 15 000, we found that poly-L-Glu and poly-L-Asp were poorer inhibitors than the shorter polypeptides. Compared to each other, however, they exerted the same degree of inhibition on Protease C1. This inhibition is not due to non-specific inhibition by any polypeptide chain since poly-L-Ser did not exert any inhibition at all. Poly-D-Glu was the most effective inhibitor of the large synthetic polyamino acids tested. Optical rotatory dispersion studies have shown that this polypeptide, like poly-L-Glu, assumes an α-helical conformation, but in the opposite screw sense [31]. Such a polypeptide might conceivably bind at the enzyme's active site with the opposite orientation. It is possible that poly-D-Glu is a better inhibitor because it binds to the enzyme, but does not serve as a substrate, whereas poly-L-Glu is not a true inhibitor, but an alternative substrate.

This type of enzyme is not exclusive to the soybean. We have found that a mung bean serine protease that has been previously characterized also digests and α' and α subunits, but not the β subunit of β-conglycinin. The enzyme generates proteolytic intermediates of the same size as the early intermediates generated by the action of Protease C1 on soybean β-conglycinin. Work to determine whether this activity is found in other legumes is under way.

EXPERIMENTAL

Plant growth. Soybean seeds (*Glycine max* [L.] Merrill, cv Amsoy 71) were purchased from Illinois Foundation Seeds (Champaign, IL). Enzyme was purified from the cotyledons of 12-day old seedlings that were grown in moist vermiculite in an environmental chamber set for 12 hr of light at 25° and 12 hr of dark at 20°.

Purification of storage proteins, Protease C1 and mung bean Protease F. β-Conglycinin was purified from soybeans using zonal isoelectric pptn chromatography as described previously [15, 32]. Purification of Protease C1 was as described in ref. [17]. The prepn obtained was 100% pure as judged by SDS-PAGE. Protease F was purified from mung bean seedling cotyledons as described in ref. [21].

Enzyme assay and generation of proteolytic intermediates. The Protease C1 degradation assay with soybean β-conglycinin was performed as previously described [16]. This assay consists of incubating the purified β-conglycinin with enzyme for a specified incubation period in 4 times concd McIlvaine citrate/phosphate buffer [33] at pH 4.5, in the presence of 0.1 mg ml^{-1} kanamycin and 0.005 mg ml^{-1} amphotericin B. The reaction is stopped by addition of SDS-PAGE sample buffer. Undigested substrate is sepd from proteolytic products by SDS-PAGE. The gels are scanned by laser densitometry and enzyme activity expressed in terms of % of substrate degraded per unit time.

The procedure to generate the proteolytic intermediates for amino-terminal sequence analysis was essentially the same as used for the Protease C1 assays except that much larger amounts of the reaction mix (from 1.50 to 4.50 ml instead of the usual 0.05 ml) were required. Multiple lanes were run on SDS-PAGE, and the gel stained with 0.3 M $CuCl_2$ for 5 min. Intermediate bands that were fairly well sepd from the others were excised, electroeluted, and concd on a Centricon concentrator with a 30 000 molecular weight cut-off. The concd sample was re-run on SDS-PAGE, blotted on PVDF membrane, lightly stained with Coomassie Blue and used for amino-terminal amino acid sequence analysis.

SDS-PAGE, electroblotting and electroelution. SDS-PAGE was done according to the method of ref. [34]. For electroblotting, the sepd peptides were transferred to Immobilon PVDF (Millipore) membrane using 10 mM Na (3-[cyclohexylamino]-1-propane-sulphonic acid) buffer–10% methanol, pH 11. The PVDF membrane was then stained with Coomassie Blue R250 (Serva Chemical Co.) (0.1% in 50% MeOH–10% HOAc). Electroelution to peptide from gel slices was performed using the Geneluter (Invitrogen) at 50 mV overnight.

Amino-terminal amino acid sequence analysis. Amino-terminal amino acid sequence analysis was done on an Applied Biosciences Model 470A gas phase protein sequencer and a model 120A PTH amino acid

on-line analyser. The analysis was carried out by Dr T. W. Thannhauser of the Analytical Chemistry and Peptide/DNA Synthesis Facility at Cornell University, Ithaca, NY.

Prediction of secondary structure at substrate cleavage site. This was done using PROSIS software (Hitachi Software Engineering, Yokohama, Japan).

Inhibition studies. Reaction mixts were set up as for enzyme assays, except for the addition of possible inhibitors such as glutamic acid and poly-L-Glu of varying average M_r. The polypeptides used as inhibitors were all from Sigma. Percent inhibition was calculated as:

$$\frac{\text{Protease C1 activity without inhibitor} - \text{Protease C1 activity with inhibitor}}{\text{Protease C1 activity without inhibitor}} \times 100$$

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