A 39 kD BARLEY SEED PROTEIN OF THE SERPIN SUPERFAMILY INHIBITS α -CHYMOTRYPSIN

by

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A 39 kD protein has been extracted from barley flour with 0.1 M monothioglycerol at pH 5.0 and purified by $(\text{NH}_4)_2\text{SO}_4$ -precipitation, anion exchange and molecular sieve chromatography. It is an N-terminally blocked, non-glycosylated, single-chain protein present in at least two molecular forms of isoelectric points 5.18 and 5.22. The amino acid composition and partial sequence analysis reveal a relationship to barley endosperm Z protein which belongs to the serpin superfamily. The 39 kD protein inhibits α -chymotrypsin while little or no effect could be demonstrated on trypsin, subtilisin, proteinase K, *S. aureus* V8 protease, thermolysin or two malt thiol endoproteinases. The 39 kD protein is immunochemically related to the major protein component in beer.

1. INTRODUCTION

The endosperm from seeds of barley and other cereals contains numerous proteins believed to act either during dormancy as inhibitors against endogenous and exogenous hydrolases (5) or play a role as amino acid storage. A relatively abundant one is the lysine-rich barley Z protein (7-11) that belongs to the serpins, a superfamily of serine proteinase inhibitors (2, 15, 16). A few serpins have either no recognized function, like chicken ovalbumin (16) and barley Z protein (11), or have evolved to mammalian hormone carriers such as thyroxine and corticoid binding globulins (4, 6) or hormone precursors such as the angiotensinogens (18). These serpins, however, are all suscep-

tible to irreversible proteolytic cleavage in the reactive center loop (2, 11, 25, 27).

We report here the isolation and preliminary characterization of a 39 kD protein present in aqueous extracts of barley flour. It is structurally related to the earlier described Z protein (7-11), but in contrast to this protein it is able to inhibit α -chymotrypsin. Both proteins are immunochemically related to the major antigenic beer protein (8, 9, 12).

2. EXPERIMENTAL

2.1. Materials

Barley seeds (*Hordeum vulgare* L., cv.s Gula and Hiproly) were obtained from Carlsberg

Abbreviations: PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecylsulfate; serpin = serine proteinase inhibitor.

Plant Breeding. Beer protein Fraction X and corresponding antiserum raised in rabbit (12) were provided by Drs. S.B. SØRENSEN (this department) and P. VAAG, Department of Biotechnology, Carlsberg Research Laboratories, respectively. Two barley malt thiol endoproteinases (19) were kind gifts of Drs. S. KOEHLER and T.-H.D. HO, Washington University, St. Louis, MO. Benzoyl-L-tyrosine ethyl ester was from Aldrich Chem. Co., Milwaukee, WI, benzoyl-L-arginine methyl ester and azocasein were from Sigma Chem. Co., St. Louis, MO. Sepharose-4B and protein A-Sepharose were from Pharmacia, Uppsala, Sweden. Proteinase K was from Merck, Darmstadt, FRG, other proteases, chemicals and materials were obtained as earlier reported (22).

2.2. Methods

2.2.1. Purification of 39 kD barley seed protein

Proteins were extracted from barley flour (2 kg) in the presence of 0.1 M monothioglycerol at pH 5.0 and subsequently fractionated by precipitation between 20-60% satd. $(\text{NH}_4)_2\text{SO}_4$ as described previously (21). Redissolved pigments adsorbed onto DEAE-cellulose at pH 7.0 while non-adsorbed proteins were applied to DEAE-cellulose DE-52 (5×21 cm), equilibrated in 15 mM Tris-HCl pH 8.0 and eluted by a gradient from zero to 0.1 M NaCl in the same buffer at a flow rate of 180 ml · h⁻¹ (21, 22). The elution of 39 kD protein and β -amylase 1 overlapped between 0.07 M and 0.08 M NaCl. The proteins were concentrated by ultrafiltration (Amicon UM 10 membrane) to 2 mg · ml⁻¹ (up to this point 0.1 M monothioglycerol was present). Portions (2 ml) were dialyzed against 75 mM NaHCO₃ pH 8.0, 5 mM dithiothreitol, made 5% in glycerol and gel filtered at room temperature on Bio-Gel P-100 (1.5×87 cm) using the same buffer and a flow rate of 8.2 ml · h⁻¹. Aliquots (approx. 0.5 mg protein) of collected fractions were dried by vacuum centrifugation and analyzed by SDS-PAGE. 39 kD protein obtained was stored in solution at either 4 °C or -20 °C prior to further characterization.

Hiprolly barley flour (700 g) was extracted essentially as above. Proteins precipitating between 45-60% satd. $(\text{NH}_4)_2\text{SO}_4$ were collected

and subjected to immunoabsorption chromatography on a conjugate (5 ml bed volume) prepared by coupling anti-beer protein Fraction X-IgG, isolated from serum using protein A-Sepharose (14), to Sepharose 4B as earlier described (24). 0.4 M NaCl, 0.01% SDS, pH 7.0 was used for sample application and washing and 0.1 M glycine, pH 2.5 for elution.

2.2.2. Isoelectric focusing and gel electrophoresis

Analytical isoelectric focusing in the range pH 3-9, 10% slab SDS-PAGE, sample denaturation and reduction, protein staining with Coomassie brilliant blue, M_r- and pI-estimation using appropriate protein marker kits were performed as earlier described (20-22).

2.2.3. Preparation and separation of tryptic fragments

39 kD protein (4 mg · ml⁻¹ in 0.1 M NH₄HCO₃ pH 8.0, 3 M urea) was incubated with trypsin (46 µg · ml⁻¹) for 2 h at 37 °C. The resulting mixture (500 µl) was lyophilized, added 0.1% trifluoroacetic acid (400 µl) and centrifuged (10000×g, 10 min). The supernatant was subjected to RP-HPLC using a Baker wide-pore C18 (RKP 7104) column (4.6×250 mm) with elution over 60 min at a flow rate of 0.5 ml · min⁻¹ by a linear gradient of 16-52% 1-propanol in 0.1% trifluoroacetic acid (23, 29). Peptides were monitored at 220 nm and material in a peak at 32% 1-propanol was rechromatographed using a shallow gradient leading to a homogeneous peptide fragment.

2.2.4. Limited proteolysis by α -chymotrypsin

39 kD protein and α -chymotrypsin were incubated for 1 h at 21 °C or 28 °C in 0.1 M Tris pH 8.0. Aliquots of the reaction mixtures were subjected to isoelectric focusing or SDS-PAGE. Prior to denaturation samples for SDS-PAGE were made 6 mM in diisopropylfluorophosphate and left at 8 °C overnight to inactivate α -chymotrypsin.

Figure 1. Separation of the β -amylase 1 (β 1 in insert) and 39 kD protein by gel filtration on Bio-Gel P-100 (see 2.2.1). β -Amylase activity was measured by incubation of 10 μ l aliquots for 30 s with 1% soluble starch essentially as earlier described (21). Fractions 25 and 26 were combined and used for the further characterization of 39 kD protein. The insert shows SDS-PAGE of the indicated amount of protein from fractions 20 (~ 10 μ g), 21-22 (~ 20 μ g), 23-24 (~ 50 μ g), 25-26 (~ 100 μ g), 27 (~ 20 μ g), 28-29 (~ 10 μ g).

2.2.5. Analytical procedures and enzyme assays

Amino acid analysis was performed on protein (10 μ g) hydrolyzed for 24, 48 and 72 h as described (29). Half-cystine was determined as cysteic acid after performic acid oxidation (13). The tryptophan content was estimated by second derivative UV spectroscopy (25). Amino acid sequencing of the 39 kD protein was performed on a Beckman automated sequencer (17, 28) and of a tryptic fragment on an Applied Biosystems Model 470A gas-phase sequencer, equipped with a Model 120 PTH analyzer. The concentration of 39 kD protein was determined either by amino acid analysis or spectrophotometrically using $E_{280}^{1\%} = 7.7$. Neutral carbohy-

drate was examined by the phenol-sulfuric acid procedure (3).

Immunodiffusion using anti-beer protein Fraction X-IgG (see 2.2.1) was performed using a 1% agarose gel in 25 mM sodium phosphate pH 7.0, 0.4 M NaCl.

Proteinase inhibition was tested by addition of 39 kD protein in 1.4-14-fold molar excess to the various enzymes (0.9 mg $\cdot$ ml $^{-1}$ in 0.1 M Tris, pH 8.0 1 mM CaCl $_2$) and assay of activity before and after this incubation using appropriate substrates as described below.

Esterase activity of α -chymotrypsin and trypsin at pH 8.0 was determined spectrophotometrically at 21 $^{\circ}$ C employing benzoyl-L-tyrosine ethyl ester and benzoyl-L-arginine methyl ester as substrates, respectively (31-33). Linear rates were followed from zero to 5 min. Endoproteinase activities of α -chymotrypsin, trypsin, subtilisin, proteinase K, *S. aureus* V8 protease, thermolysin and two thiol endoproteinases from barley malt (19) were measured with 0.1% azocasein as substrate in 0.1 M Tris-HCl, 5 mM CaCl $_2$, pH 8.0. After incubation up to 40 min at 25 $^{\circ}$ C the reaction was stopped by addition of trichloroacetic acid to 10% (w/v). The mixture was left for 30 min, centrifuged and the absorbance of the supernatant measured at 330 nm against appropriate blanks.

3. RESULTS

3.1. Purification and characterization of the 39 kD barley seed protein

The 39 kD protein was observed as a minor component in β -amylase 1-containing eluate from DEAE-cellulose chromatography (22). Fractions rich in 39 kD protein were pooled and further purified on Bio-Gel P-100 (Figure 1). Essentially pure 39 kD protein was obtained in fractions 25 and 26 (Figure 1) yielding a total of 4 mg from 1 kg of seeds. Isoelectric focusing demonstrated dominant components of pI 5.18 and 5.22 (see Figure 3). SDS-PAGE resolved the 39 kD from trace amounts of a 38 kD protein (see Figure 4, lane 1), represented perhaps by bands of pI 5.16 and 5.20 (Figure 3).

The amino acid composition of the 39 kD protein (Table I) reveals a close relationship with barley Z protein which, however, had higher pI

a.

39 kD protein S L P I R ——— M D F V A N H P F L I R E D
barley Z protein 360 M S M P L K V D L V D F V A N H P I L I R E D

↑

b.

39 kD protein Q F S M Y I L L P **E** A H D G L **S** R
248
barley Z protein Q F S M Y I L L P **G** A Q D G L **W** S

Figure 2. Sequence comparison between barley seed 39 kD protein and Z protein (11). a. The sequence C-terminal to the proteinase sensitive bond (indicated by arrow) in the serpin reactive loop (2, 11, 15). — matches inserted residues in the Z protein. b. A tryptic fragment (see 2.2.3). Differences are in bold print.

Table I. Amino acid composition of barley seed 39 kD protein

	Residues per molecule ^{a)}	Nearest integer	Barley Z protein ^{b)}
Aspartic acid	34.7	35	32
Threonine ^{c)}	19.4	19	19
Serine ^{c)}	34.8	35	34
Glutamic acid	41.1	41	44
Proline	15.1	15	14
Glycine	27.2	27	27
Alanine	36.7	37	38
Valine ^{d)}	30.8	31	31
Methionine	5.5	6	4
Isoleucine ^{d)}	16.2	16	18
Leucine	44.3	44	44
Tyrosine	7.5	8	9
Phenylalanine	20.8	21	24
Histidine	8.1	8	9
Lysine	19.4	19	20
Arginine	12.9	13	10
Tryptophan ^{c)}	4.8	5	4
Cysteine ^{f)}	1.6	2	2
Total		377	383

^a) Calculated to fit the apparent M_r

^{b)}From ref. 8

^{c)} Extrapolated to zero time hydrolysis

d) Value after 72 h hydrolysis

^e) Determined by second derivative UV spectroscopy (26)^b Determined as cysteic acid (13)

Figure 3. Isoelectric focusing of 39 kD protein. Lane 1: 39 kD protein control (3 μ g). Lane 2: 39 kD protein (28 μ M) incubated with α -chymotrypsin (48 μ M) 1 h at 28 $^{\circ}$ C (3 μ g of 39 kD protein). Lane 3: pI marker proteins.

Figure 4. SDS-PAGE of 39 kD protein. Lane 1: 39 kD protein control (5 µg). Lane 2: 39 kD protein (21 µM) incubated with α -chymotrypsin (7 µM) 1 h at 21 °C (10 µg of 39 kD protein). Lane 3: 39 kD protein incubated with α -chymotrypsin as for lane 2 (10 µg of 39 kD protein). Lane 4: α -chymotrypsin (α -Ch) (20 µg). Lane 5: M, marker proteins.

values from 5.55 to 5.8 according to an earlier report (8). The 39 kD has almost the same size as the Z protein (Table I) and is like the Z protein not a glycoprotein. Direct sequencing of the 39 kD protein (23 nmol) showed homology to the Z protein from Ser360 (Fig. 2a), which is the

Table II. Inhibition of α -chymotrypsin by 39 kD barley seed protein

39 kD : α -chymo- trypsin (mol : mol)	Esterase activity %	Endoproteinase activity %
0	100	100
1.4	89	89
3.0	40	-
7.0	41	44
14.0	20	-

The 39 kD protein and α -chymotrypsin (3 µM) were preincubated 1 h at 22 °C prior to assay of enzyme activity.

Table III. Effect of pH on the inhibitory activity of 39 kD barley seed protein

pH	Activity (% of control)
4.0	100
5.2	59
7.3	47
8.0	40
9.0	50

A three-fold molar excess of 39 kD protein was incubated with α -chymotrypsin (3 µM) at the indicated pH values for 2 h at 25 °C prior to assaying for esterase activity at pH 8.0. A separate control with α -chymotrypsin alone was incubated at each pH value and assayed. The following buffers all 1 mM in CaCl_2 were used: pH 4.0 and 5.2, 30 mM sodium acetate; pH 7.3, 30 mM 3-[N-morpholino]propanesulfonic acid; pH 8.0 and 9.0, 30 mM Tris.

N-terminus of the short C-terminal fragment. However, the yield of phenylthiohydantoin was about 2 nmol which corresponds to approx. 9% of the sample protein. Comparison with serpins suggests that it represents cleavage of the reactive center loop (2, 11, 15). The 39 kD protein was thus N-terminally blocked like the barley Z protein (11) and the majority of the isolated molecules apparently remained uncleaved. A tryptic fragment (Fig. 2b) prepared from the 39 kD protein in 60% yield shows high homology confirming that the previously determined sequence was not due to a contaminant. At present about 50% of the primary structure has been identified (LUNDGARD, SVENSSON and SVENDSEN, unpublished results) and shows 78% homology with the Z protein (11).

3.2. Interaction with α -chymotrypsin

α -Chymotrypsin converted the 39 kD protein into forms of higher pI, 5.26 and 5.33, and a polypeptide chain of 37 kD (Figs. 3 and 4). In this product (Fig. 3, lane 2, and Fig. 4, lane 3) the serpin target bond (see Fig. 2a) was hydrolyzed in greater than 90% yield as estimated from sequence analysis. A few as yet unidentified peptide bonds were cleaved to a smaller extent,

Table IV. Time course of inactivation of α -chymotrypsin by 39 kD barley seed protein

Preincubation (min)	Remaining activity (% of control)
0	100
1	100
5	74
15	48
70	39
120	40

α -Chymotrypsin (3 μ M) was incubated with three-fold molar excess of 39 kD protein in 25 mM Tris, 1 mM CaCl_2 , pH 8.0 at 22 °C. Aliquots were removed at indicated time intervals for assay of residual esterase activity.

in agreement with observations on other proteinase treated serpins (2, 27).

The 39 kD protein inhibited α -chymotrypsin (Table II), whereas a 7.8 fold molar excess only reduced the activity of trypsin by 5-10% and seemed without effect on proteinase K, *S. aureus* V8 protease, thermolysin and two barley malt thiol endoproteinases. α -Chymotrypsin inhibition was fairly constant in the range pH 5.2-9.0 and virtually not detectable at pH 4.0 (Table III). The reaction was slow with about 15 min required for loss of half the esterase activity (Table IV). The estimated second-order rate constant of the complex formation, $k_{\text{assoc}} = 10^2 \cdot 6 \cdot 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, is low, but comparable to values on physiologically relevant though relatively inefficient serpin-proteinase combinations such as α -1-proteinase inhibitor with plasmin (1, 2, 30). Isoelectric focusing or SDS-PAGE (Figs. 3 and 4) did not reveal an enzyme-39 kD protein complex in clear distinction to other serpin proteinase systems (30). Furthermore, pretreatment of 39 kD protein at pH 3.0 for 20 min at 22 °C that destroy inhibitory activity of the serpin α_1 -antitrypsin (30), or the presence of 1 mM CaCl_2 or 50 mM NaCl during incubation with α -chymotrypsin neither reduced nor enhanced the inhibition. The activity of the 39 kD protein was retained under vacuum centrifugation to dryness and redissolution, but lost by freezing of concentrated solutions or lyophilization. One might speculate, therefore, that the

earlier demonstrated lack of inhibitory activity for the barley Z protein (11) is due to inactivating pretreatment of this protein.

3.3. Immunochemical properties

Immunodiffusion was performed using 39 kD protein (2-50 μ g) and IgG (40-400 μ g) directed against beer protein Fraction X that is derived from barley Z protein (8, 12). A resulting faint precipitate, that was detected between 2-15 μ g sample and 300-400 μ g of IgG, could be slightly enhanced by pretreating the 39 kD protein with 0.5% Triton X-100 and/or heating at 80 °C for 3 min. With the same antibody barley extracts in contrast gave a sharp precipitate assumed to represent the Z protein (7, 8, 12). Immunoabsorption chromatography of a Hiproly barley flour extract (~ 150 mg protein) on anti-beer protein Fraction X-IgG-Sepharose (see 2.2.1) confirmed the low reactivity since approx. 0.5 mg of pure 39 kD protein appeared in the eluate, whereas applied purified beer protein Fraction X (5 mg), and probably also the Z protein from barley extracts, remained adsorbed. Purified 39 kD protein moreover failed to precipitate with antiserum to beer protein Fraction X in rocket immunoelectrophoresis, whereas Fraction X itself reacted strongly (data not shown). Thus, perhaps only 39 kD protein cleaved at the target-bond cross-reacts readily with Z protein and Fraction X antibodies. Similarly, it has been reported that both of two Z protein types encoded by genes on barley chromosomes 4 and 7, respectively (9) were recognized only by certain antibody preparations.

4. DISCUSSION

A 39 kD protein isolated in much smaller amounts than the Z protein (8) from aqueous extracts of barley flour also belongs to the serpin family based upon the evidence of amino acid composition, sequence homology and weak immunological cross-reactivity with the beer protein Fraction X, derived from barley Z protein (7, 12). It probably represents an earlier observed protein which is encoded on barley chromosome 7 and immunochemically related to the Z protein from chromosome 4 (9). The

proteinase inhibition involves limited proteolysis in a reactive center loop in serpins (2, 15) which in the isolated 39 kD protein has been split to a significant, but low degree. The 39 kD protein thus inhibits α -chymotrypsin, whereas proteinase inhibition could not be demonstrated for Z protein preparations, already cleaved perhaps to a far greater extent (11). The 39 kD protein, however, reacts slowly with α -chymotrypsin compared with a variety of half-times reported for association of serine proteinases with serpins (1, 2, 30), and it is uncertain whether it plays a role controlling a specific proteolytic activity related to either the plant defence against pathogens or the seed development and germination.

The serpin conformational transition from "strained" to "relaxed" (more stable) state triggered by limited proteolysis (2, 15, 25, 27) probably also occurs for the two superfamily members from barley. Thus in spite of the native 39 kD protein being sensitive to e.g. freezing it retained antigenic sites throughout the brewing processes like the Z protein, as indicated by their respective immunological cross-reactivity to the beer protein Fraction X (8, 9, 12). The knowledge of the mechanism and function of various evolutionary related serpin molecules (1, 2, 15, 27) and the present preliminary results on the interaction of 39 kD protein and α -chymotrypsin encourage the search for a target proteinase and physiological role of both this as well as the barley Z protein.

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