Arginine is essential for the α-amylase inhibitory activity of the α-amylase/subtilisin inhibitor (BASI) from barley seeds

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Treatment of barley α-amylase/subtilisin inhibitor (BASI) with reagents specific for arginine, histidine, methionine and tyrosine residues and amino and carboxyl groups indicates that an arginine residue(s) is essential for its action on the target enzyme barley α-amylase 2. Phenylglyoxal modified eight out of 12 arginine residues in BASI. Kinetic analysis shows that the inactivation of BASI follows a pseudo-first-order reaction and is due to reaction with one molecule of phenylglyoxal; the second-order rate constant is determined to be 2.95 $M^{-1} \cdot min^{-1}$. At pH 8.0, BASI and barley α-amylase 2 form an inactive 1:1 complex. The K_i value of this association is 2.2×10^{-10} M. The α-amylase protects four arginine residues and also the α-amylase inhibitory activity of BASI against phenylglyoxal. When BASI from the phenylglyoxal-modified target enzyme–inhibitor complex is isolated and subjected to a second treatment with phenylglyoxal, four additional arginine residues are modified, with concomitant loss of the inhibitory activity. These results are discussed in relation to a three-dimensional model of BASI based on the known structure of the corresponding inhibitor from wheat.

INTRODUCTION

Proteinaceous α-amylase inhibitors from higher plants have target enzymes of mammalian, insect, or plant origin [1,2]. Barley seeds contain a bifunctional α-amylase/subtilisin inhibitor (BASI) which has sequence similarity with the soybean trypsin inhibitor (Kunitz-type) family [1,3–6] and occurs in complex with the high-pI barley α-amylase (AMY2) in malt extracts [3,4]. BASI is highly specific toward cereal α-amylase of the high-pI type and neither low-pI barley α-amylase (AMY1), having 80% sequence identity to AMY2, nor α-amylases from other sources are inhibited [3,7]. BASI can form a ternary complex *in vitro* that contains both subtilisin and AMY2 [4].

The inhibitory site directed against α-amylase has not been identified in BASI or in any other proteinaceous α-amylase inhibitor. The formation of the BASI–AMY2 complex depends on ionic strength, implicating electrostatic interactions in the mechanism of inhibition [3,4,8]. Therefore, the effect of side-chain-specific chemical modification on the activity of BASI is explored in the present study. By differential labelling AMY2 is thus demonstrated to protect the activity and four arginine residues in BASI against phenylglyoxal modification, while kinetic analysis correlates the loss of inhibitory activity with modification of a single arginine residue.

EXPERIMENTAL

Materials

Barley seeds (cv. Piggy or Risø no. 1508), green malt and kilned malt (cv. Triumph or Menuet) were obtained from Carlsberg Maltings. Barley low- and high-pI α-amylase (AMY1 and AMY2) and a monocomponent form of the high-pI α-amylase family having a pI value of 5.93 (AMY2-2) were prepared as reported and the purity checked by isoelectric focusing [9–11]. DEAE-Fractogel 650S, CM-Fractogel 650M and SP-Fractogel 650M, were from Merck; Bio-Gel P-60 was from Bio-Rad; phenylglyoxal, 1-ethyl-3-(dimethylaminopropyl)carbodiimide, glycine methyl ester, iodoacetic acid, trinitrobenzenesulphonic acid, and diethyl pyrocarbonate from Sigma; tetranitromethane was from Fluka and Blue Starch from Pharmacia.

Assays

The activity of α-amylase (0.01–0.2 μM) was determined toward Blue Starch (25 mg suspended in 4 ml of 40 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$ and 0.05% BSA) at 37 °C. The reaction was stopped after 15 min by addition of 1 ml of 0.5 M NaOH. One unit of α-amylase activity corresponds to an absorbance value for the supernatant at 620 nm of 1. The inhibitory activities of BASI samples (100 μl) were assayed in 40 mM Tris/HCl containing 5 mM $CaCl_2$ and 0.05% BSA, pH 8.0, by mixing with AMY2 (0.4 μM, 100 μl) and removal of an aliquot from this mixture (25 μl) after 30 min at 37 °C for analysis of residual α-amylase activity against Blue Starch as described above. To determine a K_m value for Blue Starch initial rates of hydrolysis were determined using 10 ml samples of 11 different suspensions of starch in the concentration range 0.04–1.3% suspended in 20 mM Hepes/1 mM $CaCl_2$, pH 8.0. After addition of AMY2 (80 μl, 0.14 μM) aliquots (1 ml) were removed at 1 min intervals up to 7 min and added to 0.5 M NaOH (0.5 ml). The samples were kept on ice until centrifugation. The activity was measured spectrophotometrically as described above, the K_m determined from the initial rates using the software package GraFit. Concentrations of BASI and AMY1/AMY2/AMY2-2 were determined spectrophotometrically at 280 nm using $\epsilon_{1\%}$ values of 13 (calculated from the amino-acid composition [5]) and 24 [10] respectively. Amino-acid analysis was performed on acid hydrolysates (6 M HCl, 110 °C, 24 h) using an LKB model Alpha Plus amino-acid analyser.

Large-scale purification of BASI

BASI was isolated by an appropriate combination of published procedures [3,4,12], $CaCl_2$ (1 mM) being present at all steps. The precipitate formed at 20% satd. $(NH_4)_2SO_4$ from a 10-fold

Abbreviations used: AMY1, barley low-pI α-amylase (isoenzyme 1) family; AMY2, barley high-pI α-amylase (isoenzyme 2) family; AMY2-2, barley high-pI α-amylase 2 form having a pI value of 5.93; BASI, barley α-amylase/subtilisin inhibitor.

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concentrated (10 litre; DDS Lab-module 6000) extract of barley flour (15 kg) in 20 mM sodium acetate, pH 5.0, containing 1% (w/v) NaCl and was removed after 2 h. $(NH_4)_2SO_4$ was added to the supernatant to a resulting 70% satn. and left overnight at 4 °C. The precipitate formed was collected by centrifugation, redissolved in 20 mM sodium acetate (pH 5.0) and acetone (−20 °C) added to 60% (v/v) in an ice bath. The resulting precipitate was immediately collected, redissolved, and dialysed (Spectropor 3 tubing; molecular-mass cut-off 3500) against the above buffer. This sample was chromatographed (in two portions) on CM-Fractogel 650M (5 cm × 30 cm) using a linear gradient (2 × 1.5 litres) of NaCl (0–0.2 M) in 0.2 M sodium acetate, pH 5.0. The isolated BASI was further purified by rechromatography in the same system, followed by chromatography on DEAE-Fractogel 650S (5 cm × 30 cm) using a linear gradient (2 × 400 ml) of NaCl (0–0.3 M) in 20 mM Tris/HCl, pH 8.0. Eluate containing BASI was concentrated by ultrafiltration (molecular-mass cut-off 6000), and subjected to gel-permeation chromatography on a Bio-Gel P-60 column (2.6 cm × 90 cm) in 40 mM Tris/HCl, pH 8.0. The purified BASI was obtained at a yield of 30–40 mg/kg of flour and migrated as a single band both in isoelectric focusing, pH range 5–8, and SDS/PAGE, gradient 10–15% (w/v) acrylamide (Phastsystem, Pharmacia).

Chemical modification

Chemical modifications were performed on 20 μM BASI at room temperature if not otherwise specified and followed by determination of the inhibitory activity toward AMY2 and analysis of the content of the remaining or modified functional groups. BASI retained activity in control experiments without added reagents. Arginine residues reacted with phenylglyoxal in 50 mM Hepes, pH 8.0 [13], at 37 °C in the dark. The inhibitory activity was measured upon quenching with an excess of L-arginine. Intact arginine residues were determined by amino-acid analysis of aliquots acidified immediately after removal from the reaction mixture [14]. Carboxyl groups were modified using 1-ethyl-3-(dimethylaminopropyl)carbodiimide (40 mM) and glycine methyl ester (40 mM) in water at pH 4.75 for 2 h [15]. Sodium acetate, pH 4.8, was added at a concentration of 0.1 M to stop the reaction. Tyrosine residues reacted with tetranitromethane (5 mM) which was added in two portions at 15 min intervals in 50 mM Tris/HCl (pH 8.0) [16] in the dark. Histidine residues reacting with diethyl pyrocarbonate (1 mM) in 0.1 M Mes, pH 6.0, for 30 min were monitored spectrophotometrically at 240 nm using $\Delta\epsilon$ value of $3.2 \times 10^3\ M^{-1} \cdot cm^{-1}$ for *N*-ethoxyformylhistidine [17]. Substitution of amino groups by trinitrobenzensulphonic acid (5 mM) in 50 mM Bicine, pH 8.0, for 2 h at 37 °C [18], was quantified spectrophotometrically at 367 nm using $\Delta\epsilon_M$ $1.1 \times 10^4\ M^{-1} \cdot cm^{-1}$. Alkylation of methionine residues was attempted by adding iodoacetic acid (50 mM) in 50 mM sodium acetate, pH 4.0, and leaving the mixture for 24 h in the dark.

Protection of BASI by barley α-amylase

BASI (5 nmol) and either AMY1 or AMY2 (10 nmol) were preincubated in 20 mM Hepes/1 mM $CaCl_2$, pH 8.0, (100 μl) at 37 °C for 30 min. Phenylglyoxal (25 mM) in 62.5 mM Hepes/1 mM $CaCl_2$, pH 8.0, (400 μl) was added to the mixture from which aliquots (20 μl) were then removed at timed intervals, added to L-arginine (20 mM) in 50 mM sodium citrate/2 mM EDTA (20 μl) and incubated at 37 °C for 40 min to stop the modification and inactivate the α-amylase. In preparative-scale experiments BASI (100 nmol) and AMY2 (200 nmol) were preincubated as above in 2.2 ml of 4.6 mM Hepes/4.6 mM $CaCl_2$, pH 8.0, and mixed with phenylglyoxal (18 mM) and 2.9 ml of 85 mM Hepes, pH 8.0. After 1 h L-arginine was added to a final concentration of 24 mM. The complex of phenylglyoxal-modified BASI and AMY2 was dissociated by diafiltration (molecular-mass cut-off 6000) with repeated addition of 20 mM sodium acetate/1 mM $CaCl_2$, pH 4.7. The retentate was applied to an SP-Fractogel 650M column (1.6 cm × 25 cm) equilibrated with 20 mM sodium acetate/1 mM $CaCl_2$, pH 4.7. After washing the column the two proteins were separated by elution using a linear gradient (2 × 190 ml) of NaCl (0–0.5 M) prepared in the equilibration buffer. Fractions containing BASI (identified using SDS/PAGE; Phastsystem, Pharmacia) were pooled and concentrated. Samples were removed for analysis of activity and amino-acid content and the remainder was remodified with phenylglyoxal performed essentially as above, but with omission of AMY2.

RESULTS AND DISCUSSION

Stoichiometry of the BASI–AMY2 complex

An inactive 1:1 complex between BASI and AMY2-2 was found to be formed at pH 8.0 in the presence of BSA up to a molar ratio for BASI:AMY2 of approx. 0.9 (Figure 1a). Barley produces several members of the AMY2 isoenzyme family [19] and we find 7% uninhibited α-amylase activity (not shown) when BASI is added in 3-fold molar excess to an unfractionated sample containing all AMY2 forms. This result is perhaps due to the presence of a minor AMY2 component which is insensitive to BASI. Other workers reported less than 40% inhibition for an equimolar mixture of AMY2 and BASI at pH 8.0 [3]. The higher concentrations of BASI and barley α-amylase used in this study, as well as the addition of BSA, may explain the more efficient complex formation. Previously a 2:1 stoichiometry was found at pH 7.0 by fluorescence titration, difference spectroscopy, and gel filtration experiments [8]. In that work, however, the extent of inhibition was not measured, but a fluorescence-quenching plateau was seen at equimolar concentrations in the titration experiment and the possibility can not be excluded that inactivation of the α-amylase had occurred at the binding of the first molecule of BASI.

Replotting the data in Figure 1(a) by the method of Bieth [20] gave a slope, $K_{i(app.)}$, of 1.5×10^{-9} M (Figure 1b). By applying, however, the K_m for Blue Starch of 1.1×10^{-3} g of Blue Starch/ml

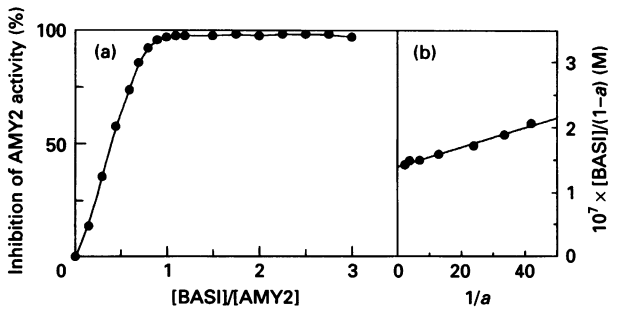

Figure 1 Inhibition of AMY2 by BASI

(**a**) AMY2-2 form (0.2 μM) was incubated with increasing amounts of BASI in 40 mM Tris/5 mM $CaCl_2$ 0.05% BSA (200 μl) at 37 °C for 30 min and the remaining activity of the AMY2-2 was determined (see the Experimental section). (**b**) Replot of data in (**a**) according to Bieth [20]. Abbreviation: *a*, relative activity of AMY2-2 in the presence of inhibitor.

Table 1 Chemical modification of BASI

BASI (20 μM) was treated with phenylglyoxal (10 mM), or a variety of different side-chain-specific reagents and the extent of modification was determined (see the Experimental section). Values in parentheses are the total number of residues present in BASI [5,8]. The inactivation of BASI was calculated from the residual inhibitory activity toward AMY2 determined relative to a BASI control incubated in the absence of reagent.

Target residue/ group	Inactivation (%)	Modified residues
Arg	97	8 (12)
COOH	42	3 (16)
Tyr	39	3 (7)
His	20	1.6 (10)
Met	9	0.1 (2)
NH_2	0	4 (7)
Trp*	0	1 (3)

* From [8].

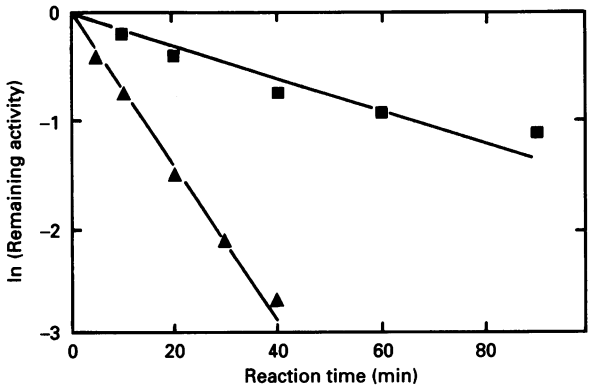

Figure 3 Inactivation of BASI with phenylglyoxal in the presence of AMY1 or AMY2

BASI (50 μM) was incubated with AMY1 (▲) or AMY2 (■) (100 μM) for 30 min at 37 °C. Phenylglyoxal was added to a final concentration of 30 mM with a concomitant 5-fold dilution of the preincubation mixture.

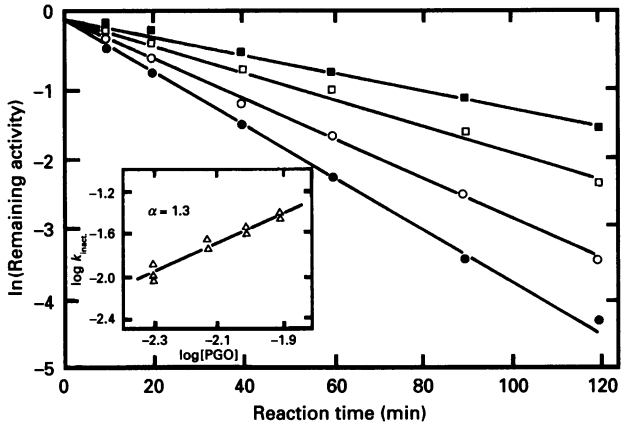

Figure 2 Inactivation of BASI at various concentrations of phenylglyoxal

BASI (20 μM) was incubated with phenylglyoxal (■, 5 mM; □, 7.5 mM; ○, 10 mM; ●, 12 mM) and the activity was determined (see the Experimental section) at the indicated time intervals. The double logarithmic plot of the obtained pseudo-first-order rate constants of inactivation, $k_{inact.}$, versus the concentration of phenylglyoxal is shown in the insert.

and assuming equilibrium has been reached between free enzyme, BASI, starch and the complexed enzyme, an intrinsic K_i of 2.2×10^{-10} M was calculated from $K_{i(app.)} = K_i(1 + S/K_m)$ [20]. The K_i value is thus 10–100-fold smaller than the value measured earlier at pH 6.0 [4], which is in accordance with a reported stronger inhibition by BASI at pH 8.0 as compared with at pH 5.5 [3]. In comparison K_d values ranging from 4.4×10^{-9} M to 3.5×10^{-11} M have been reported for other α-amylase/α-amylase inhibitor interactions [2].

Inactivation of BASI by chemical modification

When BASI was treated with reagents specific for arginine, histidine, methionine and tyrosine residues, amino, or carboxyl groups, only the modification of arginine residues caused efficient inactivation (Table 1). Eight out of the 12 arginine residues in BASI reacted with phenylglyoxal, as demonstrated by loss of arginine shown by amino-acid analysis. Similarly, amino-acid analysis of BASI indicated that all residue types except arginine were intact (results not shown) after treatment with either 2,3-butanedione or 1,2-cyclohexanedione. Reagents with different side-chain-specificity modified carboxyl groups, tyrosine or histidine residues, accompanied by 42, 39 and 20% inactivation, while trinitrophenylation of four amino groups had no effect on the activity and methionine residues were barely modified. *N*-Bromosuccinimide has previously been shown to oxidize one of the three tryptophan residues without significant inactivation [8]. BASI has no free thiol groups, but contains two disulphide bridges [5]. Taken together the results indicate that arginine is a key residue in BASI for the inhibition of AMY2. Different α-amylase inhibitors seem to possess distinctly different functional groups. Chemical modifications of inhibitors of mammalian α-amylases from *Streptomyces* [21] and plants [22–25], which are all structurally unrelated to BASI, thus have revealed arginine, tyrosine and tryptophan residues and amino groups, arginine and tryptophan residues respectively, to be involved in the inhibitory activity.

Kinetic analysis of the inactivation of BASI by phenylglyoxal

The loss of activity by modification of BASI at various concentrations of phenylglyoxal followed pseudo-first-order kinetics (Figure 2). The second-order rate constant, k, is 2.95 $M^{-1} \cdot min^{-1}$. Since k values of 19 [13] and 12 $M^{-1} \cdot min^{-1}$ [26] are reported for phenylglyoxal inactivation of ATP citrate lyase and Ap_4A phosphorylase respectively, the essential arginine residue in BASI is of normal reactivity. The inactivation of BASI was also first-order with respect to phenylglyoxal and because the slope of the double-logarithmic plot of $k_{inact.}$ versus [phenylglyoxal] is 1.3 (Figure 2, insert) reaction with approx. one molecule of phenylglyoxal is responsible for the loss of activity. This is consistent with a recently described rapid formation of a stable 1:1 complex between arginine and phenylglyoxal [27], although two molecules of phenylglyoxal were previously reported to be consumed per modified guanidino group [14].

Protection of BASI

BASI inhibits AMY2, but not AMY1 [3] and the pseudo-first-order rate constants for the inactivation of BASI by phenylglyoxal in the presence of AMY1 and AMY2 were estimated to be 0.07 and 0.01 min^{-1} respectively (Figure 3). The former value

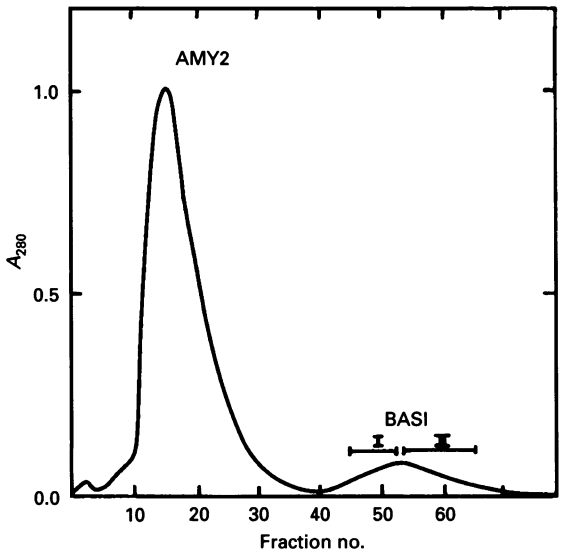

Figure 4 Separation of BASI and AMY2 on an SP-Fractogel 650M column following phenylglyoxal-treatment

BASI (2 mg) and AMY2 (9 mg) were complexed, treated with phenylglyoxal and separated after dissociation as described (see the Experimental section). See text and Table 2 for further details on pools I and II.

Table 2 Properties of phenylglyoxal-modified BASI

Modified BASI from pools I and II (Figure 4) was analysed for activity and arginine content (see the Experimental section). The retained activity is given as a percentage of that of unmodified BASI. The number of phenylglyoxal-modified arginine residues were deduced from the remaining arginine determined by amino-acid analysis. The number of AMY2-protected groups, ΔArg, is determined by subtraction of arginine contents measured before and after the second phenylglyoxal modification. *n* represents the number of residues per molecule of BASI.

Phenylglyoxal-modified BASI	Pool	Modified Arg (*n*)	ΔArg (*n*)	Activity (%)
AMY2-protected	I	3.5	–	100
	II	3.6	–	100
Remodified	I	7.5	4.0	4.5
	II	7.3	3.7	5.5

is close to 0.06 min^{-1}, the value calculated from the second-order rate constant using the equation $k \times [\text{phenylglyoxal}] = 2.95 \times 0.02\ min^{-1}$. This clearly demonstrates that AMY2 specifically protects the essential arginine residue in BASI, while the closely related AMY1, having about 80% identical amino-acid residues to the AMY2 sequence, does not afford any protection.

For differential modification BASI was first treated with phenylglyoxal in the presence of AMY2, followed by separation of the proteins on an SP-Fractogel 650M column (see the Experimental section). BASI eluted in a broad peak which was collected in two parts (pools I and II; Figure 4). Apparently relatively few versions of modified BASI resulted, since BASI from both pool I and pool II lacked about four arginine residues (Table 2). Because the isolated phenylglyoxal-derivatized BASI retained all its inhibitory activity towards AMY2 the enzyme had afforded efficient protection of the inhibitory site. However, a second treatment with phenylglyoxal resulted in a further loss of four arginine residues as well as of the inhibitory activity (Table 2). Based on kinetic evidence (Figure 2) one molecule of phenylglyoxal inactivates BASI, therefore three of the four arginine residues protected by AMY2 may not be essential for the activity of BASI. These residues might be either inaccessible to phenylglyoxal at the BASI–AMY2 interface or have acquired altered reactivity due to a change of conformation concomitant with the complexation. Alternatively, inactivation may result from conversion of any of two, three or four of the different arginine residues protected by AMY2. Although identification of the protected arginine residues does not help in distinguishing these possibilities, it allows for mutational analysis of the role of the individual arginine residues, which will be carried out using a yeast expression system for cDNA encoding BASI currently being developed in our laboratory (J. Abe, J. Christiansen, M. Søgaard and B. Svensson, unpublished work).

In the case of tendamistat, an inhibitor from *Streptomyces* which is specific towards mammalian α-amylase, a postulated essential Arg-19 is sandwiched between Trp-18 and Tyr-20. α-Amylase binding is proposed to involve a buried salt linkage between Arg-19 and a carboxylate group in the enzyme [28]. Despite the different polypeptide chain folding patterns of BASI and tendamistat [27,29], they may act by a common mechanism. In the amino-acid sequence of BASI arginines at positions 61, 75, 81 and 127 are thus flanked by aromatic and/or large hydrophobic residues. Arginine residues 61, 75 and 81, however, seem unlikely to be involved in inhibition of AMY2, since the corresponding residues in the homologous inhibitor from wheat [29,30] are near the presumed protease-binding site in the crystal structure, and BASI can inhibit subtilisin and AMY2 simultaneously [4]. On the other hand BASI arginine residues 106, 107, 127 and 155 are attractive candidates based on the model of the wheat inhibitor [29], even though Arg-155 is adjacent to Asp-156, since these two side-chains point away from each other and Phe-154 and Leu-157 make a hydrophobic environment for Arg-155 [29].

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REFERENCES

1 Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987) Oxford Surv. Plant Mol. Cell Biol. **4**, 275–336
2 Whitaker, J. R. (1989) in Food Proteins Proc. Protein Co-prod. Symp., pp. 354–380, Am. Oil Chem. Soc., Champaign, IL, USA
3 Weselake, R. J., MacGregor, A. W. and Hill, R. D. (1983) Plant Physiol. **72**, 809–812
4 Mundy, J., Svendesen, I. and Hejgaard, J. (1983) Carlsberg Res. Commun. **48**, 81–90
5 Svendsen, I., Hejgaard, J. and Mundy, J. (1986) Carlsberg Res. Commun. **51**, 43–50
6 Leah, R. and Mundy, J. (1989) Plant Mol. Biol. **12**, 673–682
7 Mundy, J., Hejgaard, J. and Svendsen, I. (1984) FEBS Lett. **167**, 210–214
8 Halayko, A. J., Hill, R. D. and Svensson, B. (1986) Biochim. Biophys. Acta **873**, 92–101
9 Svensson, B., Mundy, J., Gibson, R. M. and Svendsen, I. (1985) Carlsberg Res. Commun. **50**, 15–22
10 Gibson, R. M. and Svensson, B. (1986) Carlsberg Res. Commun. **51**, 295–308
11 Ajandouz, E. H., Abe, J., Svensson, B. and Marchis-Mouren, G. (1992) Biochim. Biophys. Acta **1159**, 193–202
12 Yoshikawa, M., Iwasaki, T., Fujii, M. and Oogaki, M. (1976) J. Biochem. **73**, 765–773
13 Ramakrishna, S. and Benjamin, W. B. (1981) Biochem. J. **195**, 735–743
14 Takahashi, K. (1968) J. Biol. Chem. **243**, 6171–6179
15 Lin, T. Y. and Koshland, Jr., D. E. (1969) J. Biol. Chem. **244**, 505–508
16 Sokolovsky, M., Riordan, J. F. and Vallee, B. L. (1966) Biochemistry **5**, 3582–3589

17 Roosemont, J. L. (1978) Anal. Biochem. **88**, 314–320
18 George, Jr., A. L. and Borders, Jr., C. L. (1986) Biochim. Biophys. Acta **569**, 63–69
19 Jones, R. L. and Jacobsen, J. V. (1991) Int. Rev. Cytol. **125**, 49–88
20 Bieth, J. (1974) in Bayer-Symposium V Protein Inhibitors (Fritz, M., Tschesche, M., Greene, L. J. and Truscheit, E., eds.), pp. 463–469, Springer Verlag, Berlin
21 Arai, M., Oouchi, N., Goto, A., Ogura, S. and Murao, S. (1985) Agric. Biol. Chem. **49**, 1523–1524
22 Kutty, A. V. M. and Pattabiraman, T. N. (1986) J. Agric. Food Chem. **34**, 552–557
23 Kutty, A. V. M. and Pattabiraman, T. N. (1985) Indian J. Biochem. Biophys. **22**, 155–160
24 Shivaraj, B. and Pattabiraman, T. N. (1981) J. Biochem. **193**, 29–36
25 Kotaru, M., Yeh, H. Y., Yoshikawa, H., Ikeuchi, T., Iwami, K. and Ibuni, K. (1989) J. Nutr. Sci. Vitaminol. **35**, 71–80
26 Robinson, A. K. and Barnes, L. D. (1991) Biochem. J. **279**, 135–139
27 Konishi, K. and Fujita, M. (1987) Biochemistry **26**, 8496–8502
28 Pflugrath, J., Wiegand, G., Huber, R. and Vértesy, L. (1986) J. Mol. Biol. **189**, 383–386
29 Zemke, K. J., Muller-Farhnow, A., Jany, K.-D., Pal, G. P. and Saenger, W. (1991) FEBS Lett. **279**, 240–242
30 Maeda, K. (1986) Biochim. Biophys. Acta **871**, 250–256

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