Volume 309, number 1, 68–72 FEBS 11467 August 1992

The amino acid sequence of a 20 kDa bifunctional subtilisin/α-amylase inhibitor from brain of rice (*Oryza sativa* L.) seeds

Ken-Ichi Ohtsubo[a] and Michael Richardson[b]

[a]*Hokuriku National Agricultural Experimental Station, Ministry of Agriculture Forestry and Fisheries, Inada 1-2-1, Joetsu, Niigata 943-01, Japan* and [b]*Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK*

Received 20 March 1992; revised version received 15 July 1992

A 20 kDa bifunctional inhibitor of the microbial proteinase, subtilisin, and the α-amylase from the larvae of the red flour beetle (*Tribolium castaneum*) was purified from bran of rice seeds by saline extraction, precipitation with ammonium sulphate, ion-exchange chromatography on DEAE-Cellulose and Toyopearl CM-650, and preparative HPLC on Vydac C_{18}. The complete primary structure was determined by automatic degradation of the intact, reduced and *S*-alkylated protein, and by manual DABITC/PITC micro-sequencing of peptides obtained from the protein following separate enzymic digestions with trypsin, pepsin, chymotrypsin, elastase and the protease from *S. aureus* V8. The protein sequence, which contained 176 residues, showed strong homology with similar bifunctional inhibitors previously isolated from wheat and barley which are related to the Kunitz family of proteinase inhibitors from legume seeds.

Rice bifunctional subtilisin/α-amylase inhibitor; Amino acid sequence; Homology with Kunitz inhibitor

1. INTRODUCTION

Cereal seeds have been shown to contain a wide variety of protein inhibitors of enzymes, such as proteinases and α-amylases, which have been assigned to at least seven different families on the basis of amino acid sequence similarities, location of disulphide bridges, and the position of reactive (inhibitory) peptide bonds (reviewed in [1]). The possible role of these molecules as defensive agents against the attacks of insect and microbial pests [2], and their potentially deleterious effects in human nutrition [3], have been a great stimulus for much research.

Rice (*Oryza sativa*) is one of the three most valuable cereal crops grown worldwide, yet in comparison with wheat, maize and barley from which numerous enzyme inhibitors have been characterized [1], relatively little is known about these important proteins in the seeds of this species. So far the only amino acid sequences determined have been for a 15 kDa trypsin inhibitor of the Bowman–Birk type [4], a 10 kDa putative proteinase/α-amylase inhibitor [5], and oryzacystatin, an inhibitor of cysteinyl proteinases [6].

In this paper we report the complete amino acid sequence of a rice 20 kDa bifunctional inhibitor of the microbial proteinase subtilisin and the α-amylase from larvae of the red flour beetle (*Tribolium castaneum*) which has high-sequence similarity with other bifunctional inhibitors previously isolated from wheat [7] and barley [8], which are related to the Kunitz family of proteinase inhibitors from legume seeds [1].

Correspondence address: M. Richardson, Department of Biological Science, University of Durham, Science Laboratories, South Road, Durham, DH1 3LE, UK. Fax: (44) (91) 374 3741.

2. MATERIALS AND METHODS

2.1. *Extraction and purification*

Bran was obtained from seeds of rice (*Oryza sativa* L.) grown in the experimental paddy fields of the Hokuriku National Agricultural Experiment Station, and polished to 90% of milling yield. 2 kg of the bran was defatted with acetone (−20°C) and extracted (×2) for 1 h at 4°C with 50 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl (8 l). After centrifugation (10,000 × *g*, 15 min, 4°C), proteins in the supernatant were precipitated with ammonium sulphate (90% saturation) and collected by centrifugation. The proteins were dissolved in distilled water and dialyzed against 50 mM phosphate buffer, pH 7.5. Insoluble proteins were removed by centrifugation and the solution applied to a column (15 × 30 cm) of DEAE-Cellulose equilibrated in the same buffer. The non-adsorbed fraction was collected and adjusted to pH 6.5 with HCl and applied to a column (2.6 × 40 cm) of Toyopearl CM-650 (Tosoh Ltd., Tokyo). The non-adsorbed fraction was collected and dialyzed against 50 mM sodium citrate buffer, pH 4.2. Insoluble proteins were removed by centrifugation and the supernatant applied to a column (2.6 × 40 cm) of Toyopearl CM-650 equilibrated in the same buffer. After washing to remove non-adsorbed proteins the column was eluted with a linear gradient of 0–1.0 M NaCl (200 ml of each) in the same buffer. The fractions containing inhibitory activity against subtilisin were pooled, dialyzed against distilled water and lyophilized. 70 mg of the partially purified proteins were dissolved in 2 ml of 0.1% aqueous TFA containing 6 M guanidine HCl, and applied to a preparative RP-HPLC column (22 mm × 25 cm) of Vydac C_{18} (218TP1022, Technicol Ltd.) which was eluted with a linear gradient of 0–50% acetonitrile in 0.1% aqueous TFA.

2.2. *Assay of subtilisin inhibition*

Inhibition of subtilisin activity was measured by adding 50 μl of sample solution to 10 μl of subtilisin (subtilisin Carlsberg, Sigma; 0.33

Published by Elsevier Science Publishers B.V.

mg/ml) in 1 ml of 0.1 M Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$. After pre-incubation for 15 min at 30°C, 1 ml of the substrate containing 0.5% (w/v) azoalbumin (Sigma) in 0.1 M Tris-HCl, pH 7.5, was added and the mixture incubated at 30°C for 15 min. The reaction was stopped by the addition of 3 ml of a 5% (w/v) solution of trichloroacetic acid. After standing for 30 min the undigested (insoluble) proteins were removed by centrifugation. 1 ml of 10% (w/v) NaOH was added to 2 ml of each supernatant and the absorbances measured at 500 nm.

2.3. *Assay of trypsin inhibition*

Samples were assayed for possible inhibitory activity against bovine trypsin by using BAPNA as the substrate [9].

2.4. *Assay of α-amylase inhibition*

The α-amylase from *Tribolium castaneum* was extracted from 20 g of defatted lyophilized larvae in 80 ml of 50 mM sodium acetate buffer, pH 4.6, containing 1 mM $CaCl_2$ and 20 mM NaCl at 2°C for 1 h. After the removal of insoluble materials by centrifugation (16,000 rpm, 15 min), cold ethanol (30 ml) was added to the supernatant, and the precipitate which formed was removed by a further centrifugation. To the supernatant (90 ml), 9.4% (v/v) of a 2% glycogen solution was added and the mixture stirred for 30 min. The glycogen–α-amylase precipitate was removed by centrifugation, suspended in 1 ml of the extraction buffer (pH 4.6) and dialyzed against the same solution for 2 h at room temperature. After removal of the glycogen the dialysate was used as the crude *Tribolium* α-amylase. Barley α-amylase was partially purified from a commercial source (Sigma) using the method described in [10]. Assays of α-amylase activity were performed using soluble starch as the substrate, and by following hydrolysis by the iodine method [11], or production of reducing sugars by the Nelson method [12].

2.5. *Electrophoresis*

The molecular weights of proteins were estimated by the SDS-PAGE method of Laemmli [13] with 15% gels using a molecular weight kit (Pharmacia-LKB).

2.6. *Reduction and S-alkylation*

Samples used for enzymatic digestions were reduced and *S*-carboxymethylated as described in [14].

2.7. *Amino acid analysis*

Samples of the reduced and *S*-carboxymethylated protein, and the peptides derived from it by enzymatic digestions, were hydrolyzed with 5.6 M HCl containing 0.02% (v/v) cresol at 108°C for 24 h. The amino acids in the hydrolyzates were derivatized with PITC and analyzed by HPLC using the Waters Pico-Tag method [15].

2.8. *Sequence determination*

The N-terminal sequence of the protein was determined using a model 477A automatic pulsed, liquid-phase protein sequencer (Applied Biosystems Ltd.) employing a standard Edman degradation sequenator programme.

Samples (3–5 mg) of the reduced and *S*-carboxymethylated protein were digested separately with trypsin, chymotrypsin, pepsin, elastase and the GLU-specific protease from *S. aureus* V8 as described in [16]. The peptides derived from these digests were purified by RP-HPLC on an analytical column (4.6 mm × 25 cm) of Vydac C_{18} (218TP54, Technical Ltd.) using gradients of acetonitrile in 0.1% (v/v) aqueous TFA. The sequences of the peptides were determined using either the manual DABITC/PITC double coupling method [17], or the automated sequencer (for certain larger peptides).

2.9. *Sequence comparisons*

The amino acid sequence of the rice subtilisin/α-amylase inhibitor was compared with those of other proteins stored in the US National Biomedical Research Foundation Databank (1990) by computer analysis using the FASTP and RDF2 programmes [18].

3. RESULTS AND DISCUSSION

Ion-exchange chromatography of rice bran proteins on Toyopearl CM-650 at pH 4.2 yielded a single major peak of inhibitory activity against subtilisin. Examination of the proteins present in this peak by SDS-PAGE revealed the presence of four major bands with M_r values of approximately 35, 20, 15 and 10 kDa. These proteins were readily separated by RP-HPLC on a preparative column of Vydac C_{18}. The second major peak, which was eluted at an acetonitrile concentration of 30%, was found to contain a mixture of proteins which strongly inhibited bovine trypsin and had M_r values of about 15 kDa. The N-terminal sequences of these proteins were shown to be MERPWK, which is identical to the sequence previously reported for the rice 15 kDa trypsin inhibitor of the Bowman–Birk type [4]. The bulk of the inhibitory activity against subtilisin was located in the third major peak (37% acetonitrile) which was found to contain a homogeneous protein of M_r 20 kDa. This protein gave 100% inhibition of subtilisin at molar ratios of 1:1 enzyme:inhibitor, which is similar to the results observed for related proteins from wheat, Triticale and rye [19]. It was also a strong inhibitor of the α-amylase from the larvae of the red flour beetle (*Tribolium castaneum*), but had no detectable inhibitory activity against barley α-amylase even when present in large (10×) molar excess.

The amino acid sequence of this bifunctional subtilisin/α-amylase inhibitor from rice (RASI) which was determined by a combination of automated and manual sequencing methods is shown in Fig. 1. The sequence was in agreement with the results of amino acid analyses (not shown), and the M_r value of 18,745 calculated from the sequence is close to the estimate of 20 kDa made from the results of SDS-PAGE. Low levels of microheterogeneity were detected in positions 69 (traces of A in place of S) and 83 (traces of P in place of F), and a minor form of the protein was present in which one of the R residues normally found in positions 37 and 38 was deleted.

The sequence shown for RASI in Fig. 1 is identical with the first 24 amino acids previously reported as the N-terminal sequence of a rice subtilisin inhibitor [20], except in positions 9 (E in place of Q) and 12 (E in place of Q). Similar bifunctional inhibitors of subtilisin and α-amylase with M_r values of 19–20 kDa have also been reported previously from other cereals, such as barley [8,21,22], wheat [7,23] and rye [24], but it should be noted that these inhibitors appeared to be specific for the endogenous plant α-amylases and none were reported to inhibit an enzyme from an insect such as *Tribolium*. Comparison of the RASI sequence with those of the wheat (WASI) [7] and barley (BASI) [8] inhibitors (Fig. 2) reveals that the three proteins are clearly homologous and have approximately 60% sequence identities. This finding is somewhat surprising

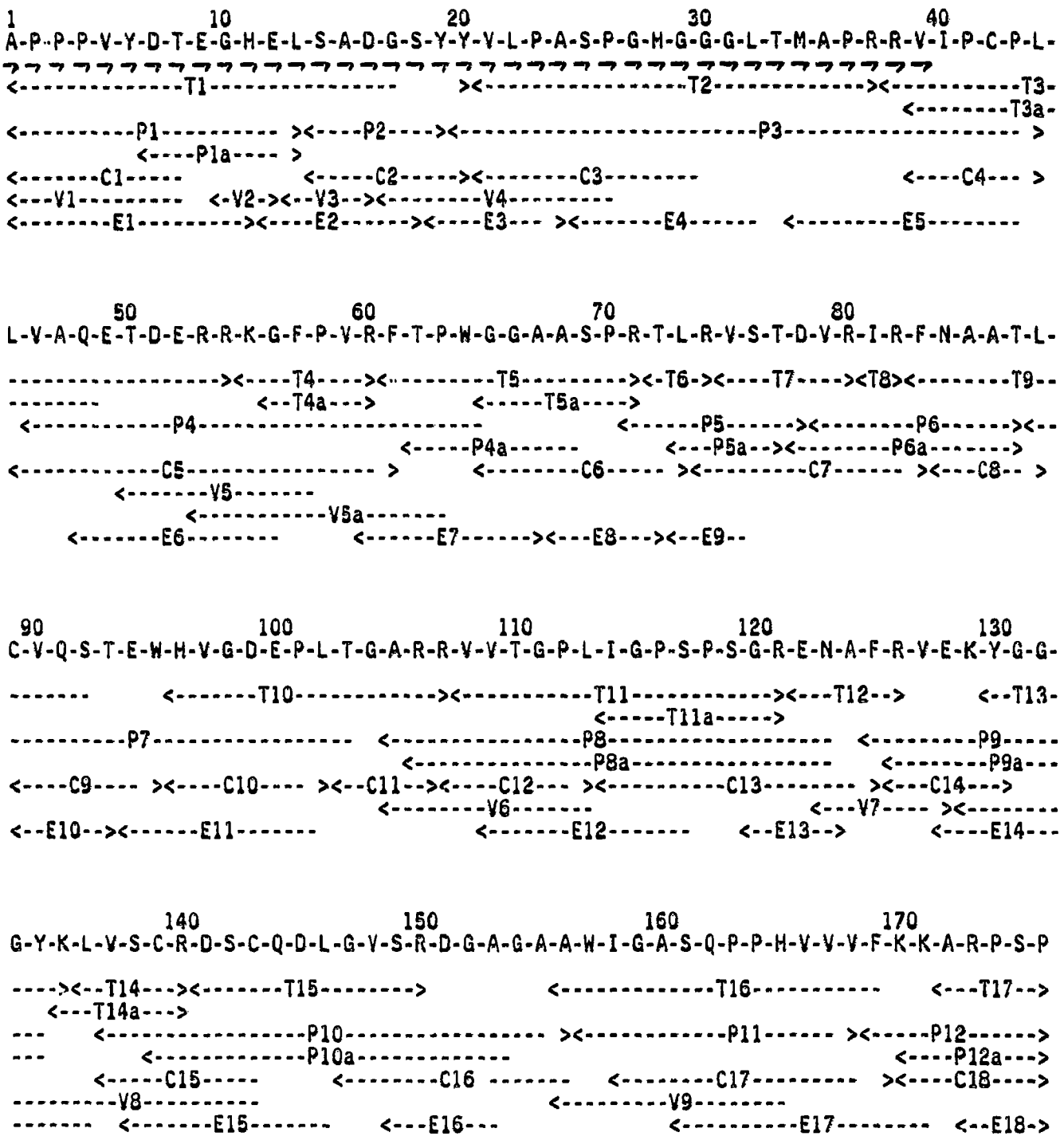

Fig. 1. The amino acid sequence of the bifunctional inhibitor of subtilisin and *Tribolium castaneum* α-amylase from seeds of rice (*Oryza sativa*). Dotted lines indicate peptides obtained from digestions with trypsin (T), pepsin (P), chymotrypsin (C), the V8 protease from *S. aureus* (V) and elastase (E) which were sequenced by the manual DABITC/PITC method or by automated means. Arrows (→) indicate residues determined by automatic degradation of the intact reduced and *S*-carboxymethylated protein.

as Weselake et al. [25] were unable to detect any protein with immunological identity to the BASI inhibitor when they analyzed extracts of rice seeds by the Ouchterlony double-diffusion method.

A computer search of the US National Biomedical Research Foundation Databank also revealed that RASI has statistically significant (z values >15) sequence identities with a number of Kunitz-type proteinase inhibitors from different legume seeds [26–30], and similarities to a nodulin protein from senescent

```
           1        10        20        30        40        50        60
RASI       APPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRRVIPCPLLVAQETDERRKGFPVR
WASI       DPPPVHDTDGNELRADANYYVLPANRAHGGGLTMAPGHGRRCPLFVSQEADGQRDGLPVR
BASI       ADPPPVHDTDGHELRADANYYVLSANRAHGGGLTMAPGHGRHCPLFVSQDPNGQHDGFPVR
             **** ** * ** **  **** *  **********     *** * *       * * *

                  70        80        90       100       110       120
RASI       FTPWGGAASPRTLRVSTDVRIRFNAATLCVQSTEWHVGDEPLTGARRVVTGPLIGPSPSGR
WASI       IAPHGGAPSDKIIRLSTDVRISFRAYTTCVQSTEWHIDSELVSGRRHVITGPVRDPSPSGR
BASI       ITPYGVAPSDKIIRLSTDVRISFRAYTTCLQSTEWHIDSELAAGRRHVITGPVKDPSPSGR
             * * * *    * ******* * * * * *******   *   * * * ***   *******

                 130       140       150       160       170
RASI       ENAFRVEKYGGG----YKLVSCRDSCQDLGVSRDGAGAAW-IGASQPPHVVVFKKARPSP
WASI       ENAFRIEKYSGAEVHEYKLMACGDSCQDLGVFRDLKGGAWFLGATEPYHVVVFKKAPPA
BASI       ENAFRIEKYHGAEVSEYKLMSCGDWCQDLGVFRDLKGGAWFLGATEPYHVVVFKKAPPA
           ***** *** *      ***  * * ******* **  * **  **  * **********
```

Fig. 2. Comparison of the amino acid sequences of the rice bifunctional inhibitor of subtilisin and α-amylase (RASI) with WASI, the subtilisin/α-amylase inhibitor from wheat [7] and with BASI, the barley subtilisin/α-amylase inhibitor [8]. The numbering of residues refers only to RASI –, indicates deletions inserted in sequences to maximize homology. *, indicates residues which are invariant in all sequences shown.

nodules of winged bean (*Psophocarpus tetragonolobus*) [31], an albumin from cocoa seeds (*Theobroma cacao*) [32], a cathepsin D inhibitor from potatoes [33], the taste-modifying protein, miraculin [34], and sporamin from sweet potato [35].

Other inhibitors of α-amylases from insect pests have been previously reported from other cereal seeds, such as barley [36], maize [37], wheat [38,39], sorghum [40,41] and Job's Tears (*Coix lachryma-jobi*) [42]. However, none of these proteins appears to be structurally related to the rice 20 kDa RASI protein, the majority belonging to the cereal superfamily of 12–13 kDa inhibitors [1].

The precise function(s) of RASI and the structurally related WASI and BASI proteins in the seeds of rice, wheat and barley remains unclear. The fact that they are strong inhibitors in vitro of microbial enzymes, such as subtilisin and the proteinases from *Aspergillus* spp. [19] suggests that they may act in the defence of the seeds against invasion by microorganisms. Similarly as the rice RASI protein inhibits the α-amylase of *Tribolium* larvae, it is tempting to speculate that it may also be involved in defence against insect predation [1,2]. Some research has suggested that the levels of α-amylase inhibitors in wheat seeds may represent a valid measure of resistance to post-harvest insects [43]. However, it should be noted that although the 13 kDa α-amylase inhibitors from wheat [38,39] were found to be potent in vitro inhibitors of the gut enzymes from the larvae of both insect species, *Tribolium confusam* and *Callosobruchus maculatus*; when incorporated into artificial diets they only inhibited larval development and increased larval mortality in *T. confusam* [44].

Acknowledgements: We are grateful to the Japanese Science and Technology Agency and the Japanese Ministry of Agriculture, Forestry and Fisheries for financial support. We also thank Mr. J. Gilroy for invaluable technical assistance.

REFERENCES

[1] Richardson, M. (1991) in: Methods in Plant Biochemistry, vol. 5 (Rogers, L. ed.) pp. 261–307, Academic Press, London.
[2] Ryan, C.A. (1990) Annu. Rev. Phytopathol. 28, 425–449.
[3] Friedman, M. (1986) Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods, Plenum Press, New York.
[4] Tashiro, M., Hashino, K., Shiozaki, M., Ibuki, F. and Maki, Z. (1987) J. Biochem. 102, 297–306.
[5] Yu, Y.G., Chung, C.H., Fowler, A. and Suh, S.W. (1988) Arch. Biochem. Biophys. 265, 466–475.
[6] Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S. (1987) J. Biol. Chem. 262, 16793–16797.
[7] Maeda, K. (1986) Biochim. Biophys. Acta 871, 250–256.
[8] Svendsen, I., Hejgaard, J. and Mundy, J. (1986) Carlsberg Res. Commun. 51, 43–50.
[9] Kanamori, M., Ibuki, F., Tashiro, M., Yamada, M. and Miyoshi, M. (1975) J. Nutri. Sci. Vitaminol. 21, 421–428.
[10] Silvanovich, M.P. and Hill, R.D. (1975) Anal. Biochem. 73, 430–433.
[11] Bird, R. and Hopkins, R.H. (1954) Biochem. J. 56, 86–89.
[12] Nelson, N. (1944) J. Biol. Chem. 153, 375–377.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[15] Millipore Waters Chromatography Corp. (1984) Operators Manual No. 88140.
[16] Aitken, A., Geisow, M.J., Findlay, J.B.C., Holmes, C. and Yarwood, A. (1989) in: Protein Sequencing: A Practical Approach (Findlay, J.B.C. and Geisow, Eds.) pp. 43–68, IRL Press, Oxford.
[17] Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978) FEBS Lett. 93, 205–214.

[18] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[19] Mosolov, V.V. and Shulgin, M.N. (1986) Planta 167, 595–600.
[20] Kato, I., Tominaga, N. and Kihara, F. (1972) in: Proc. Conf. Protein Structure, vol. 23 (Iwai, K., ed.) pp. 53–56, Maebashi Pub.
[21] Mundy, J., Svendsen, I. and Hejgaard, J. (1983) Carlsberg Res. Commun. 48, 81–90.
[22] Yoshikawa, M., Iwasaki, T., Fuji, M. and Oogaki, M. (1976) J. Biochem. 79, 765–773.
[23] Mundy, J., Hejgaard, J. and Svendsen, I. (1984) FEBS Lett. 167, 210–214.
[24] Shulgin, M.N. and Mosolov, V.V. (1985) Biokhimiya (Eng. trans) 50, 1676–1684.
[25] Weselake, R.J., MacGregor, A.W. and Hill, R.D. (1985) Cereal Chem. 62, 120–123.
[26] Richardson, M., Campos, F.A., Xavier-Filho, J., Macedo, M.L.R., Maia, G.M.C. and Yarwood, A. (1986) Biochim. Biophys. Acta 872, 134–140.
[27] Yamamoto, M., Hara, S. and Ikenaka, T. (1983) J. Biochem. 94, 849–863.
[28] Joubert, F.J., Heussen, C. and Dowdle, E.B.D. (1985) J. Biol. Chem. 260, 12948–12953.
[29] Kim, S., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K. and Kaizuma, H. (1985) J. Biochem. 98, 435–448.
[30] Negreiros, A.N.M., Carvalho, M.M.M., Xavier-Filho, J., Blanco-Labra, A., Shewry, P.R. and Richardson, M. (1991) Phytochemistry 30, 2829–2833.
[31] Manen, J.F., Simon, P., Van Slooten, J.C., Osteras, M., Frutiger, S. and Hughes, G.J. (1991) Plant Cell 3, 259–270.
[32] Spencer, M.E. and Hodge, R. (1991) Planta 183, 528–535.
[33] Mares, M., Meloun, B., Pavlik, M., Kostka, V. and Baudys, M. (1989) FEBS Lett. 251, 94–98.
[34] Theerasilp, S., Hitotsuya, H., Nakajo, S., Nakaya, K., Nakamura, Y. and Kurihara, Y. (1989) J. Biol. Chem. 264, 6655–6659.
[35] Hattori, T., Nakagawa, T., Maeshima, M., Nakamura, K. and Asahi, T. (1985) Plant. Mol. Biol. 5, 313–320.
[36] Lazaro, A., Sanchez-Monge, R., Salcedo, G., Paz-Ares, J., Carboner, P. and Garcia-Olmedo, F. (1988) Eur. J. Biochem. 172, 129–134.
[37] Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) Nature 327, 432–434.
[38] Silano, V., Furia, M., Gianfreda, L., Macri, A., Palescandolo, R., Rab, A., Scardi, V., Stella, E. and Valfre, E. (1975) Biochim. Biophys. Acta 391, 170–178.
[39] Kashlan, N. and Richardson, M. (1981) Phytochemistry 20, 1781–1784.
[40] Bloch Jr., C. and Richardson, M. (1991) FEBS Lett. 279, 101–104.
[41] Bloch Jr., C. and Richardson, M. (1992) Protein Seq. Data Anal. 5 (in press).
[42] Ary, M.B., Richardson, M. and Shewry, P.R. (1989) Biochim. Biophys. Acta 993, 260–266.
[43] Yetter, M.A., Saunders, R.M. and Boles, H.P. (1979) Cereal Chem. 56, 243–244.
[44] Gatehouse, A.M.R., Fenton, K.A., Jepson, I. and Pavey, D.J. (1986) J. Sci. Food Agric. 37, 727–734.