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2D-HPLC SEPARATION, ELECTROPHORETIC CHARACTERIZATION AND *N*-TERMINAL SEQUENCES OF OAT SEED PROLAMINS

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Key Word Index—*Avena sativa*; Graminae; oat, seed; isolation; characterization; prolamins; alcohol-soluble proteins.

Abstract—In common with the alcohol-soluble seed storage proteins (also called prolamins) of other cereals, oat avenins are a series of polymorphic molecules belonging to a multigenic family. By using ion-exchange followed by reverse phase HPLC, all the proteins of oat grain soluble in ethanol–water (9:11) have been isolated and purified. They were checked by urea and SDS-PAGE, characterized by *N*-terminal sequencing and identified by searching in sequence libraries. Beside avenins, the true prolamins, three other low-M_r proteins, soluble in ethanol–water, were observed, two of them were identified as α-amylase/trypsin inhibitors which are found in the endosperm of other cereals, and the third one as a novel protein. The microheterogeneity of true avenins are revealed by *N*-terminal sequencing, although half of them are blocked to Edman degradation. Like other prolamins, avenins exhibit short tandem repeats, heptapeptides slightly different from those found in the Festucoideae subfamily. Their predicted secondary structure reveals successive β-turns which might be arranged in a pseudo-helix structure. In agreement with this arrangement, the hydropathy profile strongly suggests that these pseudo-helices could be associated in a super-secondary structure analogous to that described for maize zein, a structure well-fitted to maximal packing of amino acids in the reserve tissues of the seed.

INTRODUCTION

Among oat seed storage proteins, avenins, the oat prolamins, form a peculiar class of proteins which are soluble in ethanol–water solutions and have been classified in three sub-groups (α to γ) according to their decreasing electrophoretic mobility in urea-PAGE [1]. Avenins are stored in the starchy endosperm of the caryopsis within protein bodies [2] and their only known biological function is for the storage of nitrogen for the young seedling when it is still heterotrophic. The amino acid content of avenins [1, 3], like other prolamins, shows a high proportion of proline and glutamine residues and a deficiency in some essential amino acids (mainly lysine)[4]. Among oat seed proteins, the relative abundance of avenins is *ca* 10%, a value quite low compared to the proportion of prolamins in wheat or barley in which it may reach 45%. Avenins also belong to a multigenic family, but their heterogeneity is not as pronounced as in other cereals (wheat, maize, barley) [4–6]. Like other prolamins, they are used as genetic markers because their expression in the grain is only governed by genetic factors and not by growing conditions [6, 7], except in the case of acute starvation in some elements [8]. The phylogeny of the genus *Avena* has therefore been studied through investigations about avenin inheritance [9, 10]. In the absence of data on tertiary structure, structural predictions from *N*-terminal sequences of crude [11] or partially purified [12] avenins contributed to a better tentative understanding of the functional packing of prolamins.

This paper describes a rapid and efficient method for the isolation of all the alcohol-soluble oat proteins by 2D HPLC (ion-exchange and reverse phase). Purified protein fractions were characterized by their *N*-terminal sequences, identified in sequence libraries, and by their electrophoretic mobilities in two different systems, acidic urea and SDS-PAGE. Calculated structural features have also been investigated to understand how point mutations of the avenin structure would affect optimal storage of prolamins within protein bodies.

RESULTS

Isolation and identification of alcohol-soluble proteins

The cation-exchange chromatography of crude avenins is shown in the first dimension of Fig. 1. It can be divided into three parts: the first three peaks which were not retained in the column by ionic interactions; peaks 4 to 14, eluted by a low ionic strength (between 5 and 80 mM NaCl); peaks 15 and 16, eluted at the end of the gradient (0.7 M NaCl) All peaks were sufficiently resolved to be collected with a very low level of cross contamination. Each of these fractions was chromatographed on a reverse phase column (second dimension of Fig. 1). Only peaks containing proteinaceous material are shown (fractions 1 and 2 were not resolved and consequently eliminated). For some of the ion-exchange fractions, the second separation gave two (fractions 8, 10 and 15) or three peaks

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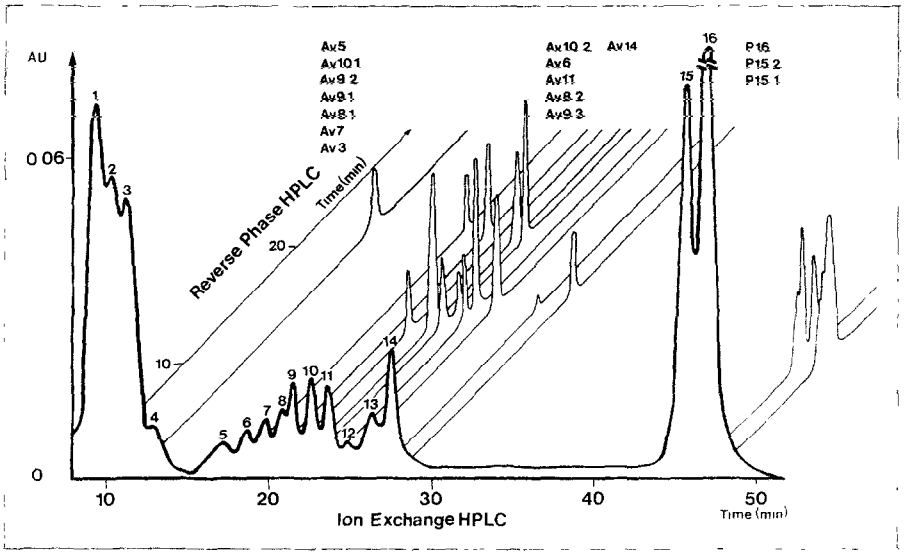

Fig 1 2D-HPLC of oat alcohol-soluble proteins Elution monitored using absorbance at 275 nm First dimension ion-exchange chromatography on TSK SP-5PW Second dimension reverse phase-HPLC on Aquapore (C8) RP 300

(fraction 9). Except for fractions 15 and 16, which appear more hydrophilic, the polarity of proteins is similar to each other and they were all eluted between 34.5 and 37.5% MeCN

The electrophoretic diagrams of the fractions collected after the second chromatography are shown in Fig. 2. Fractions P 15 1, P 15.2 and P 16 exhibit a behaviour very different from the other proteins· they have a low M_r 13 000, 15 000 and 16 000, respectively) and urea-PAGE, run for a shorter time (2 hr), shows that they have a quite different net charge at acidic pH, in agreement with their behaviour in ion-exchange chromatography Considering the group of proteins eluted at low ionic strength, they have been identified as true avenins and classified using urea-PAGE The fast-moving avenins α_2, contaminated by α_1, are present in peaks Av 13 and Av 14 Their M_r is 24 500 The γ_1 avenins are found in fractions Av 6, Av 8.1 and Av 9 1 (respectively 23 000, 27 000 and 26 000). The fraction Av 7 is the only one to contain a γ_2 constituent of 28 000, whereas both fractions Av 8 2 and Av 9 3 are made up of γ_3 avenins of 25 500 and 24 500, respectively. The fractions Av 5, Av 10 1 and Av 11 are γ_4 avenins of 24 000, 32 000 and 30 000, respectively Fraction Av 3 is a γ_5 avenin of 25 000 and peaks 9 2 and 10 2 are γ_6 avenins of 31 500

N-*Terminal sequence of avenins*

Table 1 summarizes, together with the compilation of M_r and denominations of the proteins, the *N*-terminal amino acids of the purified fractions. It emphasizes that, among true avenins, nearly half of the proteins are blocked to Edman degradation (Av 3, Av 5, Av 8 2, Av 9.2, Av 9 3 and Av 10 2) The *N*-terminal sequences of other proteins are reported in Fig 3, where they are classified in order to emphasize internal homologies The comparison among the *N*-termini of true avenins reveals highly homologous regions, as has been found in other prolamins [4] Although they all share in common the same *N*-terminus, one can distinguish avenins with an asparagine instead of an aspartic acid at position 7 At position 20 begins a repetitive structure made of heptapeptide repetitive units (*PFVQQQQ*) with some variations either in number of peptide units or in point mutation of some isolated residues, including insertions and deletions. Seven of them are observed in the longest determined sequence (Av 10 1) The fraction Av 6 is more peculiar, in that PFV in the repetitive unit is replaced by MLL This sequence has been first identified as a contaminant of a blocked protein, because the level of the initial sequencing yield was quite low (11%) As it shows clear differences with those of other avenins, and that Av 7, Av 10 1 and Av 11, which have a labile Asp-Pro bond at position 7, have been found to be contaminated by their own sequence beginning at position 8, Av 6 is assumed to be a blocked *N*-terminal avenin with a partially cleaved Asp-Pro bond near the *N*-terminus All the purified avenins have a high sequence homology with the already known avenins [12] In contrast, the low M_r proteins show very different sequences The homology of P 15 1 and P 15 2 with the wheat α-amylase inhibitor CIII [13] and the maize trypsin/factor XIIa inhibitor [14], respectively are shown in Fig 3

Secondary structure of avenins and hydropathy profiles

The secondary structure prediction of the *N*-terminal of avenins is constant from one sequence to another and is not affected by point mutations It can be described as repetitive β-turns beginning at position 9, following a *N*-terminal end in extended structure (results not shown) The repetitive peptide units are predicted to be four residues in a β-turn separated by three residues in aperiodic structure The prediction of the peculiar peptide of Av 6 gives a lower prediction of turns The hydropathy profiles of avenins exhibiting rapid alternance of hydrophilic and hydrophobic areas are shown in Fig 4 The primary structure of the repetitive units results in such

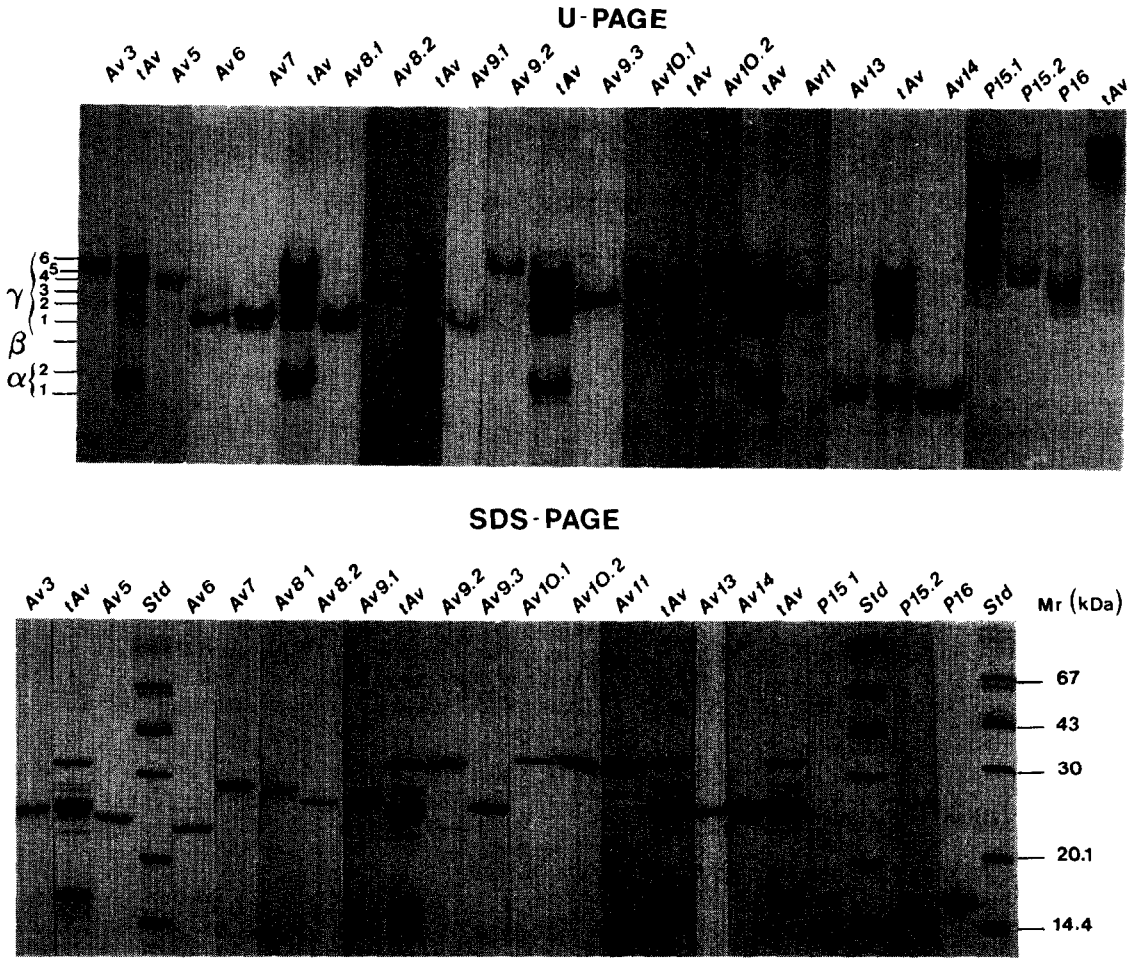

Fig. 2. Urea and SDS-PAGE of 2D-HPLC fractions. Fractions are numbered according to Fig. 1 and Table 1. tAv total alcohol-soluble proteins, α, β and γ avenin nomenclature [1] M_r standards were purchased from Pharmacia (LMW)

peculiar alternances in phase with the secondary structure, whatever the type of repetitive peptide.

DISCUSSION

Except for the rare β-avenins, all the proteins observed in the electrophoretic patterns of oat alcohol-soluble proteins have been isolated and most of them purified to homogeneity by a 2D chromatographic procedure which is more efficient than a single reverse phase chromatography [15]. This procedure confirms that avenins, which are eluted between 34 5 and 37 5% of MeCN, are less hydrophobic than other prolamins [16]. As has been found in other cereals [17], oat seed contains some alcohol-soluble proteins quite different from prolamins and related to trypsin/α-amylase inhibitors. Beside these proteins, the fraction P 16 appears as a novel one, which can be classified neither as a true prolamin nor as an enzyme inhibitor. It has recently been sequenced as a cDNA clone and revealed to have homology with oat globulins [18] Contrary to prolamins, these proteins are partially extracted by a high ionic strength, in the absence of EtOH when oat flour is submitted to sequential extraction [19].

The oat prolamins, called 'true avenins' in this article, are somehow different from the prolamins of other cereals. Thus contrary to Triticeae prolamins [4], they constitute a single continuous class ranging from 23000 to 32000. Two slightly different N-terminal sequences have been identified for avenins, independent from their class and M_r, except that of Av 6 which can be classified separately Its MFLQQQQ repeat could only be found in low M_r avenins, while the wide-spread consensus peptide unit PFVQQQQ, with sometimes deletion or insertion of one glutamine, is observed in all kinds of avenins. The existence of only one type of N-terminal sequence for all avenins, whatever their subgroup, α or γ, shows that they belong to a quite homogeneous family. Nevertheless, due to large differences in M_r, the commonly used sub-group classification [1] is relevant The consensus sequence is not identical to those of prolamins of the Triticeae tribe (barley hordeins [20, 21], wheat gliadins [22–30] and rye secalins [29]), of the Panicoideae subfamily [4, 16, 31], or of the grasses [32]. Only a 44% homology can be observed in the 11 N-terminal residues with sequence of γ-gliadin [29]. In addition, it is noticeable that half of the avenins exhibit a blocked N-terminus, which has only be signaled in barley B-hordeins [33]. Homologies revealed

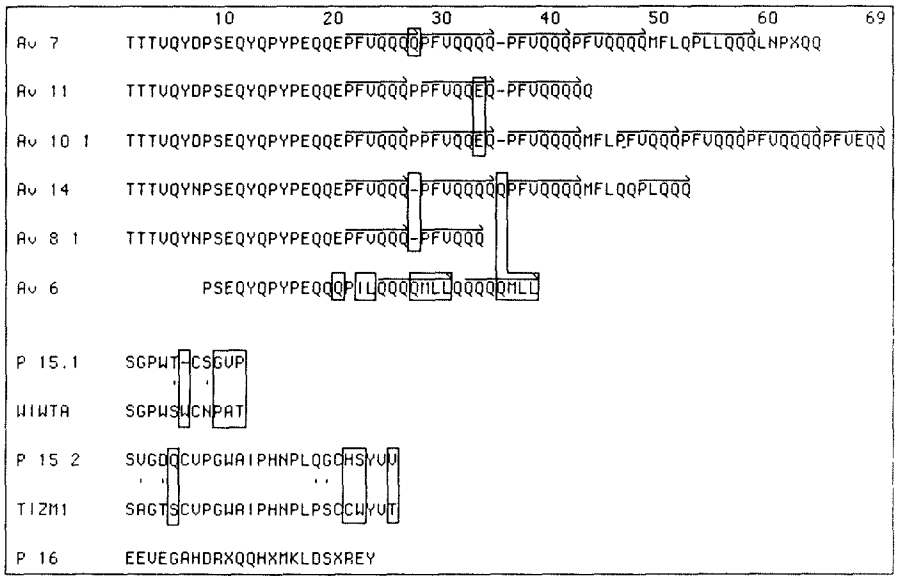

Fig. 3 *N*-terminal sequences of 2D-HPLC fractions. Differences have been emphasized by boxes up to position 40 (the alterations have not been boxed further because of the numerous insertions) WIWTA and TIZM1 are the *N*-terminal ends of the wheat α-amylase inhibitor CIII [25] and of the maize trypsin/factor XIIa inhibitor [26], respectively. Vertical bars between P 15 1 and WIWTA and P 15 2 and TIZM1 indicate structurally equivalent residues [46]

Table 1 Identification and classification of 2D-chromatographic fractions

Fraction	Protein class*	M_r (kDa)	*N*-Terminus
Av 3	γ 5	25	blocked
Av 5	γ 4	24	blocked
Av 6	γ 1	23	Partially blocked (Pro)
Av 7	γ 2	28	Thr
Av 8 1	γ 1	27	Thr
Av 8.2	γ 3	25.5	blocked
Av 9 1	γ 1	26	not determined
Av 9.2	γ 6	31 5	blocked
Av 9 3	γ 3	24 5	blocked
Av 10 1	γ 4	32	Thr
Av 10.2	γ 6	31 5	blocked
Av 11	γ 4	30	Thr
Av 13	α 2	24 5	not determined
Av 14	α 2	24 5	Thr
P 15 1	Hydrolase inhibitor	13 5	Ser
P 15 2	Hydrolase inhibitor	15	Ser
P 16	Unknown	16	Glu

*α, β and γ avenin nomenclature [1]

by the comparison of *N*-terminal sequences between oat prolamins and other cereal prolamins strongly suggest only one common ancestral gene of the β-gliadin type [12] for avenins and explains why the sequencing of crude avenins [11] did not reveal observable microheterogeneity.

Three *N*-terminal sequences (A, E and F) of less

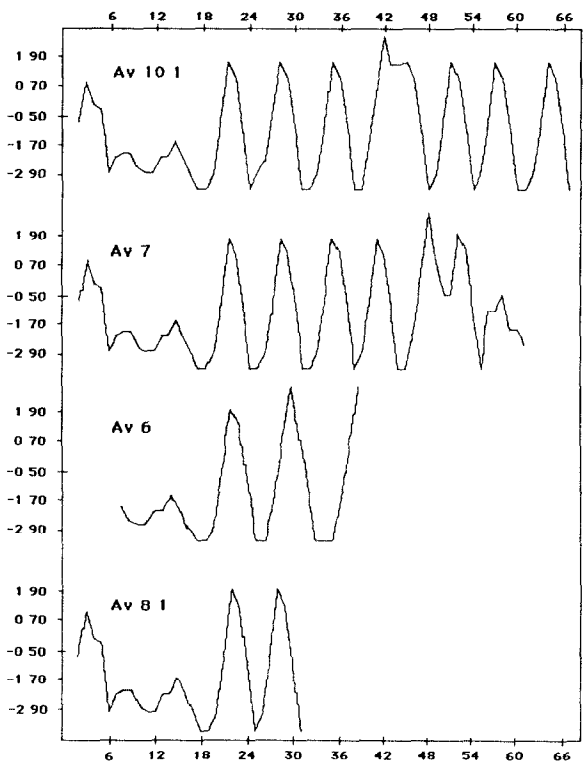

Fig 4 Hydropathy profiles of avenin *N*-termini Computed according to Kyte and Doolittle [48] with a window width of 3 residues *X*-axis aminoacid position, *Y*-axis hydropathy index

purified avenin fractions have already been reported [12]. Although related to a γ_4 avenin, avenin A appears to be close to fraction Av 6, a γ_1 avenin, but could not be identified among the presently purified avenins. Due to its M_r and its blocked N-terminus, fraction A should be the fraction Av 5. Avenin E is undoubtedly assigned to Av 14, an α_2 avenin, and avenin F to Av 10.1, rather than to Av 11, because of its greater M_r. The complete sequence of a purified avenin has been recently published [34]. This protein, named N9, has a M_r and N-terminal sequence corresponding to an α_2 avenin with only two mutations at positions 46 and 47. It is therefore analogous to Av 14.

A possible conformation of the repetitive peptide unit can be assumed from secondary structure prediction and hydropathy. As for prolamins from other species [4, 12], the avenin tandem repeats are predicted in successive β-turns which might be arranged in a pseudo-helix structure [35]. In agreement with this arrangement, the hydropathy profile strongly suggests that these pseudo-helices could be associated in a super-secondary structure formed by pseudo-helices associated together along their hydrophobic generatrix (corresponding to lines formed by phenylalanines and valines), the hydrophilic moiety (mainly glutamines) facing towards the solvent. This structure would be analogous to that described for zein, the maize prolamin, which is predicted in α-helix barrels [31, 36]. It would favour a compact well-fitting arrangement of prolamins so as to increase the storage of amino acids in the smallest possible volume and therefore explain the evolutionary conservation of such structures for prolamins. The selective pressure could have resulted from steric features involved in the protein–protein interaction.

EXPERIMENTAL

Protein extraction. Mature naked oat seeds (*Avena nuda*, cultivar Rhea) were supplied by the INRA Plant Breeding Station of Rennes. Oat grains were ground in liquid N_2 with a 0.25 mm sieve. The meal was defatted twice for 1 hr at room temp. in H_2O-satd *n*-BuOH. Prolamins were extracted with EtOH–H_2O (9:1) (w/w) at room temp. according to ref. [1]. A solvent:flour ratio of 10:1 (w/w) was used and the mixture was continuously magnetically stirred, before centrifugation (1000 *g*, 30 min, 20°). The supernatant was lyophilized prior to further investigations.

HPLC. A Spectra Physics chromatographic system composed of a 8700 XR LC pump, a 8750 organizer and a 8773 XR UV detector was used. All solvents (UV grade) were continuously degased with He. Samples, dissolved in the starting solvent (1.6 mg/ml), were manually injected through a 500 μl-loop and the elution performed at room temp. (22°). Cation exchange-HPLC was performed with a TSK SP-5PW (7.5 × 75 mm) column (Toyo Soda) using solvents A (35 mM citric acid, 30 mM disodium phosphate and 30% MeCN, pH 3.4) and B (solvent A with 0.7 M NaCl). The following gradient was applied: 100% of A from 0 to 7 min, from 0 to 15% of B between 7 and 37 min, and finally from 15 to 100% of B, at a constant flow rate of 0.8 ml/min. Reverse phase-HPLC, adapted from ref. [37] was conducted with an Aquapore (C8) RP 300 (Brownlee Labs) cartridge (4.6 × 30 mm) using the solvents A (15% MeCN–85% H_2O with 0.1% TFA) and B (40% MeCN–60% H_2O with 0.1% TFA). The applied gradient was 100% of A from 0 to 3 min, 0 to 70% of B between 3 and 8 min and from 70 to 100% of B between 8 and 28 min at a constant flow rate of 1 ml/min. Elution was monitored through absorbance at 275 nm. Protein contents of collected peaks were determined by the method of ref. [38].

PAGE. Two different kinds of electrophoresis, urea and SDS-PAGE, were used to characterize the HPLC fractions. Urea-PAGE was performed with a running gel containing 6% acrylamide, 0.2% N,N′-Methylene bisacrylamide and 6 M urea at pH 3.6. The running buffer was a pH 3.6 soln containing 6 M urea, 2.45 g/l Al lactate and 9 ml/l of a soln containing 90% lactic acid. Protein samples were dissolved in a sample buffer containing 8 M urea, 1.22 g/l Al lactate and 34 ml/l of a soln containing 20% lactic acid. Gels were run for 15 min at 150 V (constant voltage) and then for 3 hr 45 min at 410 V (constant voltage). The gels were stained with a soln containing 12% TCA and 5% of an ethanolic soln of Coomassie Serva Blue (7 mg/ml) and destained with water. The procedure of ref. [39], modified in ref. [40], was used for SDS-PAGE. The running gel contained 12.6% acrylamide, 0.33% bisacrylamide, 0.1% SDS in a 375 mM Tris–HCl buffer, pH 8.9. The stacking gel contained 4% acrylamide, 0.1% bisacrylamide, 0.1% SDS in a 125 mM Tris–HCl buffer, pH 6.8. Running buffer was a soln containing 50 mM Tris, 384 mM glycine and 0.1% SDS, pH 8.5. Protein samples were dissolved in a sample buffer containing 10 mM Tris, 1 mM EDTA, 0.0054 N HCl, 10% sucrose and 0.25% SDS, pH 8. Samples were heated in boiling water for 5 min before electrophoresis. Gels were run for 15 min at 46 mA (constant current) and then for 2 hr at 94 mA (constant current). The gels were stained according to ref. [41] with a Coomassie Serva Blue soln in EtOH–HOAc–H_2O (3:1:6) and destained according to ref. [42] with a MeOH–HOAc–H_2O (5:7.5:87.5). Low M_r calibration kits were provided by Pharmacia.

Amino acid sequencing. N-Terminal sequences were determined with a 470A Applied Biosystems gas phase sequenator linked to a 120A PTH-analyzer according to ref. [43]. A 500 picomol deposit of protein was used. A current initial yield of 0.22 to 0.55 was observed.

Protein research in data libraries. The FASTP algorithm [44] was used to screen the NBRF protein sequence library (release 13) together with the programme of ref. [45] utilizing the similarity matrix of ref. [46], also used to screen the GENPRO library, corresponding to the open reading frames of Genbank (release 50), accessible in the BISANCE database, at CITI2 in Paris.

Prediction of secondary structure and hydropathy. Secondary predictions were established using the algorithm of ref. [47], with all decision constants put to zero, and the hydropathy profiles computed with the programme of ref. [48] with a window width of 3 residues.

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