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CHARACTERIZATION OF EXTRACELLULAR PROTEASES FROM *TRAMETES TROGII*

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Abstract—The peptidase activities excreted in culture broths of *Trametes trogii* mycelium have been identified by determining the digestion pathway of various peptides. Insulin β-chain (30 residues), procasomorphin (10 residues) and two peptides of five residues (proctolin and thymosin α_1 fragment 23–27) were utilized as model substrates. Aminopeptidase, carboxypeptidase, endopeptidase and dipeptidyl aminopeptidase activities were revealed and information on their specificity was deduced. Preliminary data on the pH-dependent activity of the peptidases were also obtained by sequence analysis of the fragment mixtures produced at different pH values.

INTRODUCTION

The characterization of extracellular enzymes produced by basidiomycete fungi during growth can be very useful to study their metabolism as well as to obtain information on the degradation pathways of the natural polymers that fungi use as nutrients. While the production and characterization of enzymes such as phenol oxidases, peroxidases and cellulases have been reported [1–7], little information on proteolytic enzymes is available [8]. Proteinases produced by animal and plant species are utilized in a variety of industrial and diagnostic applications [9]; thus, a search for new proteolytic enzymes may provide material for diverse end uses. The analysis of the complete set of proteinases excreted in the culture medium by growing cells could represent a complex and time-consuming task, if purification of the proteolytic enzymes and/or fractionation of the peptides produced by digestion of a protein substrate have to be carried out. For this reason, alternative procedures should be preferred, which allow the collection of useful information without performing any protein purification and/or peptide fractionation. For instance, fast atom bombardment mass spectrometry (FAB-MS) analysis performed on unfractionated fragment mixtures was an advantageous tool in the study of peptide digesion [10]. Petrilli *et al.* used an alternative approach in investigating the resistance of an opioid peptide to the action of gut proteases from a plant-eating insect [11]; the aim of Petrilli's study was to evaluate the possibility of introducing in plants the gene(s) encoding neuropeptide(s) able to interfere with insect metabolism and to confer resistance to the pest. This procedure, not requiring any purification step, is based upon the possibility of deducing the sequence of the fragments produced at different digestion times by means of the automatic Edman degradation carried out on the unfractionated peptide mixtures. The method can be considered of general utility and utilized for several goals. In fact, sequence data being quantitative, the determination of the amount of each fragment in the mixture furnishes information on the specificity of the hydrolytic enzymes. In the present study, we applied this approach to identify the peptidases excreted in culture broths by *Trametes trogii* and to collect some data on their catalytic properties; in particular, we determined the combined action of these enzymes on model peptides of different length and sequence, assessing their hydrolysis pathways at distinct pH values.

RESULTS AND DISCUSSION

Incubation and sequence analyses

Proctolin, thymosin α_1 fragment 23–27, insulin β-chain and procasomorphin were incubated at 37° with culture broth of *Trametes trogii*. Incubations were performed at both pH 8 and pH 5 and aliquots of the digestion mixtures corresponding to 1500–2000 pmol of the original peptides were withdrawn at different times and submitted to automatic sequence analysis. The main problems in the interpretation of the sequence analysis of peptide mixtures are due to the carryover of the Edman reaction and to the different yield of phenylthiohydantoin (PTH)-amino acids; it is well known that the yield of the Edman degradation lowers at progressive steps of the sequence

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Table 1. Sequence analysis of the alkaline mixture of proctolin peptide (1500 pmol of original peptide) after 1 hr incubation at pH 8. Peptide sequence is shown at the bottom. (A) Amounts of all PTH-amino acids obtained at each step of the Edman degradation; the data of the identified residues are in **bold**. (B) Residues identified at each step and their amounts data. (C) Sequences of the fragments present in the mixture

(A) Edman steps

	Residues (pmol)																		
	D	N	S	Q	T	G	E	A	H	Y	R	P	M	V	C	W	F	I	K	L
1	10	5	11	2	11	23	5	17	2	**293**	**836**	**32**	3	9	7	9	6	8	6	**415**
2	7	4	4	1	**18**	11	5	9	1	**873**	58	**219**	1	7	6	7	2	7	2	**163**
3	5	3	2	1	**103**	7	3	5	—	38	13	**155**	—	4	2	4	2	7	1	**803**
4	3	2	1	—	**92**	6	2	3	—	6	9	**524**	—	2	—	3	1	4	1	19
5	3	1	1	—	**289**	3	2	3	—	3	9	98	—	—	—	2	1	3	1	6

(B) Residues identified at each step

1		2		3		4		5	
R	(836)	Y	(873)	L	(803)	P	(524)	T	(289)
Y	(293)	L	(163)	P	(155)	T	(92)		
L	(415)	P	(219)	T	(103)				
P	(32)	T	(18)						

(C) Sequences of the fragments present in the mixture

(1) R–Y–L–P–T intact peptide; approx 900 pmol
(2) Y–L–P–T fragment 2–5; approx 180 pmol
(3) L–P–T fragment 3–5; approx 250 pmol
(4) P–T fragment 4–5; approx 30 pmol
Additional free amino acids (first step): R_1, Y_2, L_3.

1 2 3 4 5
Peptide sequence: R Y L P T.

analysis and is not equal for each PTH-amino acid. However, with the advent of the modern pulsed-liquid phase sequencers equipped on-line with PTH analysers, the carryover has been well quantified and, despite the yield problems, the identification of several amino acid residues at each step requires just a little care and experience. Thus, if a peptide of known sequence is digested by any agent(s), it is possible to assess the sequence and amount of each fragment present in the hydrolysis mixture.

Proctolin peptide

Table 1 shows the information obtained from the sequence analysis of the alkaline digestion mixture of proctolin (about 1500 pmol of the original peptide) after 1 hr incubation. The mono-literal code sequence of proctolin is reported at the bottom. Data of all PTH amino acids obtained at each of the five steps of the Edman degradation are shown in Table 1(A). The residues present at each step were easily identified on the basis of the pmol data shown in **bold**. These residues and their pmol data are listed in Table 1(B), while the deduced sequences of the fragments present in the mixture are indicated in Table 1(C). The sequence of the intact peptide was present, together with the sequences of the fragments 2–5, 3–5 and 4–5; as a consequence, the released free amino acids (portions of R_1, Y_2 and L_3) are present at the first step. This kind of hydrolysis clearly demonstrates the action of an aminopeptidase, and information on its kinetic properties can be obtained looking at the amount of the residues identified at each step. The amount of all individual fragments can be evaluated from the data of those residues which can belong only to the sequence of a single fragment. For example, the amount of the intact peptide can be estimated as about 900 pmol (60% of the original sample) since Y at the second step (873 pmol), L at the third step (803 pmol), P at the fourth step (524 pmol) and T at the fifth step (289 pmol) are not in common with the sequence of other fragments; on the contrary, the pmol value of R at the first step being due to the contribution of both the intact peptide and the free arginine released in the mixture, is not a good parameter to estimate the amount of the intact peptide; moreover, the yield of PTH-arginine is quite low. The N-terminal arginine was hydrolysed slowly, whereas the tyrosine present at the second position was removed faster. In fact, while the amount of the fragment 2–5 can be estimated as about 12% of the original sample, the fragment 3–5 is about 17% of the original sample. The leucine present at the third position and preceding a proline residue was hardly removed, as the fragment 4–5 was present in traces. In synthesis, this enzyme hydrolyses basic residues slowly, is quite effective on the removal of hydrophobic residues but is not efficient in the hydrolysis of X-proline peptide bonds. The results obtained from the sequence analysis of

Table 2. Sequence analysis of the alkaline mixture of proctolin peptide (1500 pmol of original peptide) after 4 hr incubation at pH 8. Peptide sequence is shown at the bottom. (A) Amounts of all PTH-amino acids obtained at each step of the Edman degradation; the data of the identified residues are in **bold**. (B) Residues identified at each step and their amounts data. (C) Sequences of the fragments present in the mixture

(A) Edman steps

	Residues (pmol)																		
	D	N	S	Q	T	G	E	A	H	Y	R	P	M	V	C	W	F	I	K	L
1	10	7	17	2	**22**	25	6	24	2	**401**	**882**	**74**	3	14	3	5	8	9	8	**632**
2	9	6	9	2	**36**	14	4	14	1	**431**	64	**334**	1	9	1	3	4	7	3	**172**
3	5	3	3	2	**142**	9	2	9	1	25	17	**149**	1	6	—	3	2	5	2	**275**
4	2	3	1	1	**86**	5	1	5	1	4	11	**200**	1	4	—	3	2	3	2	9
5	2	2	1	—	**79**	3	2	3	1	2	8	65	—	2	—	2	1	3	1	5

(B) Residues identified at each step

1		2		3		4		5	
R	(882)	Y	(431)	L	(275)	P	(200)	T	(79)
Y	(401)	L	(172)	P	(149)	T	(86)		
L	(632)	P	(334)	T	(142)				
P	(74)	T	(36)						
T	(22)								

(C) Sequences of the fragments present in the mixture

(1) R–Y–L–P–T	intact peptide; approx 450 pmol
(2) Y–L–P–T	fragment 2–5; approx 190 pmol
(3) L–P–T	fragment 3–5; approx 380 pmol
(4) P–T	fragment 4–5; approx 50 pmol
(5) T	residue 5; approx 30 pmol

Additional free amino acids (first step): R_1, Y_2, L_3 (P_4)

	1	2	3	4	5
Peptide sequence:	R	Y	L	P	T.

the alkaline mixture after 4 hr incubation are shown in Table 2 and confirm this conclusion. The amount of the intact peptide was still 30% in the mixture, that of the fragment 3–5 increases (25% of the total sample) and the fragment 4–5 was present in traces (3% of the total sample). The small quantity of threonine detectable at the first step (Table 2(A)) can be due to a minimal removal of the proline from the fragment 4–5 by the aminopeptidase, and/or to the action of a carboxypeptidase removing the C-terminal threonine very slowly, and/or to the activity of a dipeptidyl aminopeptidase removing a small amount of the dipeptide L-P from the fragment 3–5. Finally, we established that the aminopeptidase is not capable of hydrolysing proctolin at acidic pH. In fact, the sequence analysis of the pH 5 mixture revealed that the peptide was intact even after 4 hr incubation (not shown).

Thymosin α_1 *fragment* 23–27 *peptide*

Further information on the specificity of the aminopeptidase was obtained from the sequence analysis of the incubation mixtures of a thymosin α_1-deriving peptide, possessing a basic N-terminal residue (lysine) as proctolin, but acidic residues (glutamic acid) at second, third and fifth position of its sequence (Table 3). The sequences of the fragments (Table 3(C)) present in the alkaline digestion mixture after 1 hr incubation were deduced from the residues identified at each step (Table 3(B)) on the basis of the PTH amino acids pmol data (Table 3(A)). The amount of the intact peptide can be estimated as about 800 pmol (A at the fourth step) keeping in mind the progressive lowering of the yield of the Edman degradation. The hydrolysis of the basic N-terminal residue was slow also in this case, whereas the removal of the glutamic acid at the second position was very fast. In fact, the fragment 2–5 was not present at all owing to the absence of alanine and glutamic acid at the third and fourth step, respectively. The action of a carboxypeptidase removing some of the C-terminal glutamate residue cannot be excluded since a part of the glutamic acid at the first step could be due to the presence of the free C-terminal residue in the mixture. The sequence analysis of the alkaline mixture after 4 hr incubation showed that about 30% of the peptide was still intact and that the removal of both glutamic acid and alanine at the third and fourth position was quite complete (not shown). As already assessed for proctolin, thymosin α_1 peptide was completely undigested after 4 hr incubation at pH 5 (not shown).

The results obtained with proctolin and thymosin α_1 peptides demonstrate that the specificity in the amino acid removal of the aminopeptidase at alkaline pH is as

Table 3. Sequence analysis of the alkaline mixture of thymosin α_1 peptide (fragment 23–27) 1500 pmol of the original peptide) after 1 hr incubation at pH 8. Peptide sequence is shown at the bottom. (A) Amounts of all PTH-amino acids obtained at each step of the Edman degradation; the data of the identified residues are in **bold**. (B) Residues identified at each step and their amounts. (C) Sequences of the fragments present in the mixture

(A) Edman steps

	Residues (pmol)																		
	D	N	S	Q	T	G	E	A	H	Y	R	P	M	V	C	W	F	I	K	L
1	11	3	8	2	7	5	**751**	**97**	1	1	—	5	2	6	4	9	4	6	**813**	4
2	9	2	3	1	4	4	**463**	**85**	1	1	—	5	1	5	2	7	2	5	37	4
3	7	2	2	2	2	4	**642**	15	—	1	—	4	1	5	2	7	3	5	6	3
4	5	2	2	1	2	3	71	**613**	—	—	—	2	—	2	—	6	2	4	4	3
5	5	1	1	1	2	3	**218**	49	—	—	—	2	—	1	—	6	1	2	3	2

(B) Residues identified at each step

1		2		3		4		5	
K	(813)	E	(463)	E	(642)	A	(613)	E	(218)
E	(751)	A	(85)						
A	(97)								

(C) Sequences of the fragments present in the mixture

(1) K–E–E–A–E intact peptide; approx 800 pmol
(2) E–A–E fragment 3–5; approx 100 pmol
(3) A–E fragment 4–5; approx 100 pmol
Additional free amino acids (first step): K_1, E_2, E_3 (E_4)

	1	2	3	4	5
Peptide sequence:	K	E	E	A	E.

follows: acidic residues > hydrophobic residues > basic residues. In these experiments no evidence was found for the presence of an endopeptidase activity in the culture broth of *Trametes trogii*. However, the two peptides tested are very short and their sequences could not present suitable sites for endopeptidases; thus, to have useful information on the presence of such enzymes, a larger peptide was tested.

Insulin β-chain

The sequence analysis results of the alkaline incubation mixture (30 min) of insulin β-chain with *Trametes trogii* culture broth are shown in Table 4. As can be observed by PTH amino acids pmol data (Table 4(A)), no amino acid residue was identified at the 15th step of the Edman degradation, meaning that the intact peptide was not present in the mixture and that the longest fragment produced by the digestion was 14 residues long. The commercial insulin β-chain used contains cysteine residues as cysteic acid, whose PTH derivative was not present as an internal standard of the instrument; PTH-cysteic acid was identified qualitatively only on the basis of its retention time (4.48 min). The sequences of the fragments present in the mixture are listed in Table 4(C); the residues which can belong only to the sequence of a single fragment and useful to identify it quantitatively are shown in boldface. The combined action of several enzymes in the hydrolysis of the peptide can be inferred. The large amount of alanine at the first step (600 pmol) can be due only to the presence of a carboxypeptidase removing the C-terminal residue from a large part of the peptide molecules; furthermore, a minor quantity of lysine present at position 29 could have been removed by this enzyme since this amino acid was also present at the first step (87 pmol); however, K at the first step could be common to the sequence of the fragment 29–30, whose presence in the mixture was possible, as discussed below. The action of the aminopeptidase was quite effective, the sequences of the fragments 1–14, 2–14, 3–14, 4–14, 5–14 and 6–14 being identified. In addition, the presence in the mixture of internal fragments was clearly demonstrated, indicating the action of an endopeptidase. The fragment 7–14 seems to be produced by an endopeptidase cleavage at level of the peptide bond L_6–C_7 since its amount (200 pmol) was higher than that of the fragments 5–14 and 6–14 (100 pmol) produced by the progressive action of the aminopeptidase. Endopeptidase cleavages at level of V_{12}, A_{14} and Y_{16} produced significant amounts of the fragments 13–14 and 15–16; further cleavages at level of F_{24}, L_{17}, F_{25} and Y_{26} were demonstrated by the presence of the fragments 17–24, 18–24, 26–30 and 27–30, respectively. Alternatively, some of these fragments (18–24 and 27–30) could be generated by an aminopeptidase cleavage of the parent peptides (17–24 and 26–30, respectively). Finally, the presence of the dipeptides 27–28 and 29–30 could be attributed to a dipeptidyl aminopeptidase activity on the fragment 27–30 and/or,

more unlikely, to an endopeptidase cleavage at level of P_{28} on the same fragment. Table 4(D) shows all the hydrolysis sites of the peptide identified by the enzymes present in the culture broth. As can be observed, all the sites recognized by both endopeptidase and carboxypeptidase were hydrophobic residues, while these enzymes possess little or no ability in the removal of basic residues.

The most important information obtained from the sequence analysis of the alkaline mixture after 1 hr incubation revealed that the aminopeptidase and carboxypeptidase hydrolyzed the internal fragments produced by the endopeptidase; moreover, a large amount of the fragment 7–14 was produced, confirming that the cleavage at L_6 was due to the endopeptidase (not shown). Finally, the sequence analyses of the pH 5 mixtures after 0.5, 1 and 4 hr incubation confirmed that the aminopeptidase was not effective at acidic pH and revealed that the endopeptidase and carboxypeptidase activities were less efficient (not shown).

Procasomorphin

Procasomorphin (Table 5) is a proline-rich peptide deriving from the hydrolysis of caseins [12, 13]. It is a good substrate for checking the activity of dipeptidyl aminopeptidase of type IV [10, 11], an enzyme capable of removing N-terminal X-Pro dipeptides from a polypeptide chain [12, 14–18]. Amounts of the PTH-amino acids obtained from the sequence analysis of the alkaline mixture after 2 hr incubation with *Trametes trogii* culture broth are reported in Table 5(A). The intact peptide was not present in the mixture as no amino acid was identified at the sixth step of the sequence analysis; the N-terminal valine was completely removed by the aminopeptidase and the fragment 2–10 was produced. The absence of the fragment 3–10 confirms that the aminopeptidase was not effective in the hydrolysis of X-Pro peptide bonds. However, the fragment 2–10 was digested by a typical dipeptidyl aminopeptidase of type IV. In fact, the dipeptides Y_2–P_3 and F_4–P_5 were present in large amounts and completely removed from the fragment 2–10 as can be deduced from the absence of F_4 and G_6, respectively, at the third step. The remaining fragment 6–10 was present in the mixture and a part of it was further hydrolysed. In fact, the dipeptides G_6–P_7 and I_8–P_9 were both present in the mixture; moreover, K_{10} was detectable at the first step. A portion of the lysine at the first step is likely due to the action of the carboxypeptidase; however, this enzyme is not able to remove the C-terminal proline from the fragments produced by the dipeptidyl aminopeptidase, since free proline was not detected at the first step of the Edman degradation. As assessed by the sequence analysis of the acidic mixture after 4 hr incubation, procasomorphin was not hydrolysed at pH 5 (not shown). In fact, as already observed, the aminopeptidase was not effective at acidic pH and the dipeptidyl aminopeptidase IV was not able to hydrolyse the intact peptide; moreover, the endopeptidase was not able to hydrolyse this proline-rich peptide.

Trypsin-like activity

Insulin β-chain possesses a potential site of hydrolysis for trypsin-like activities at level of the peptide bond R_{22}–G_{23} (Table 4). No hydrolysis occurred at this site since no fragment starting from G_{23} was detected in the mixture (Table 4(C)), suggesting that no trypsin-like activity was present in the culture broth. This result was confirmed by a trypsin activity assay of the culture broth using a synthetic substrate.

CONCLUSIONS

To the best of our knowledge, this is the first report describing the complete set of extracellular proteinases produced by a basidiomycete fungus. On the basis of their assessed specificity and pH-dependent activity, the enzymes excreted by *Trametes trogii* can be classified as follows: aminopeptidase type A, carboxypeptidase type A, dipeptidyl aminopeptidase type IV and thermolysin-like endopeptidase. It is reasonable that the fungus produces extracellular proteinases of broad specificity; in this way, peptides and proteins occurring in the surrounding environment can be efficiently digested. In this view, the lack of a highly-specific trypsin-like endopeptidase is not surprising. The only highly-specific enzyme was the dipeptidyl aminopeptidase IV, whose particular function in the digestion of proline-rich peptides should be necessary. This enzyme is widely distributed in mammalian tissues [12, 14–18] and culture broths of fungi could represent an accessible and abundant source for its purification devoted to different end uses.

In the last few years, mass spectrometry analysis performed on individual peptides or peptide mixtures has been widely used to assess various peptide and protein features [12, 19–26]. This paper shows that the sequence analysis of peptide mixtures performed by modern automatic sequenators represents an alternative to mass analysis and presents some advantages. Mass spectrometry data are qualitative; moreover, some failure in identifying all fragments owing to the well-documented phenomenon of signal suppression is possible [27]. Sequence data are complete and can be used for a number of goals. For example, the automatic sequence analysis of peptide mixtures, supported by suitable algorithms, was revealed to be an excellent tool in assessing protein sequences very quickly [28, 29]. This work should represent an exhaustive example of a different application, suggesting that various kinds of problems can be resolved utilizing this approach.

EXPERIMENTAL

Growth of mycelium. *Trametes trogii* mycelium was maintained at 4° and transferred monthly on fresh potato

Table 4. *Sequence analysis of the alkaline mixture of insulin β-chain (2000 pmol of the original peptide) after 0.5 hr incubation at pH 8. Peptide sequence is shown at the bottom. (A) Amounts of all PTH-amino acids obtained at each step of the Edman degradation; the data of the identified residues are in* **bold**; *PTH-cysteic acid was qualitatively identified (*) (B) Residues identified at each step and their amounts. (C) Sequences of the fragments present in the mixture; the residues which can belong only to the sequence of the individual fragments are shown in* **bold**. *(D) Insulin hydrolysis sites recognized by the enzymes present in the culture broth: ⟩ aminopeptidase; ⟨ carboxypeptidase; ↓ endopeptidase*

(A) Edman steps

	Residues (pmol)																			
	D	N	S	Q	T	G	E	A	H	Y	R	P	M	V	C	W	F	I	K	L
1	31	**88**	21	**163**	**222**	24	**119**	**563**	**58**	**1132**	13	23	13	**1478**	***	16	**1322**	18	**87**	**2066**
2	62	**344**	11	**55**	**302**	**170**	33	**144**	**96**	**340**	11	**180**	5	**1554**	***	15	110	11	12	**111**
3	41	**120**	**47**	**271**	36	**255**	68	42	**38**	38	11	**331**	2	145	***	16	23	9	**76**	**138**
4	**18**	**24**	**44**	**99**	15	**695**	**261**	**152**	**123**	16	19	97	2	35	*	17	14	7	**154**	**117**
5	16	15	**43**	23	8	**105**	**403**	**198**	**59**	11	**93**	49	1	19	*	18	14	6	29	**505**
6	12	12	**27**	13	9	**182**	41	35	**27**	10	**209**	33	1	**96**	***	15	7	5	13	**137**
7	11	10	**20**	10	7	**641**	**92**	13	**17**	11	54	21	1	**42**	*	13	**69**	4	7	**44**
8	9	10	**107**	7	6	**150**	**29**	**57**	**15**	5	18	16	—	**25**	—	15	**156**	5	4	**23**
9	9	7	**34**	5	6	31	**19**	**28**	**70**	5	10	13	—	**17**	—	14	27	5	2	**22**
10	7	8	8	5	6	11	**18**	**21**	**17**	4	6	12	—	**23**	—	13	14	4	2	**64**
11	7	5	6	4	6	10	**17**	**15**	5	3	5	11		**43**	—	15	9	5	2	**26**
12	6	5	8	4	5	10	**25**	**15**	3	3	5	11	—	**17**	—	16	6	4	2	7
13	6	6	7	5	3	8	**20**	**16**	3	2	2	9	—	4	—	15	5	4	1	5
14	5	6	6	4	3	8	6	14	2	2	1	7	—	3	—	12	5	4	1	4
15	5	4	6	4	2	8	6	5	2	2	1	7	—	3	—	13	4	4	1	4

(B) Residues identified at each step

1	2	3	4	5	6	7	8	9	10	11	12	13	14
F (1322)	V (1554)	N (120)	Q (99)	H (59)	L (137)	C (*)	G (150)	S (34)	H (17)	L (26)	V (17)	E (20)	A (14)
V (1478)	N (344)	Q (271)	H (123)	L (505)	C (***)	G (641)	S (107)	H (70)	L (64)	V (43)	E (25)	A (16)	
N (88)	Q (55)	H (38)	L (117)	C (*)	G (182)	S (20)	H (15)	L (22)	V (23)	E (17)	A (15)		
Q (163)	H (96)	L (138)	C (*)	G (105)	S (27)	H (17)	L (23)	V (17)	E (18)	A (15)			
H (58)	L (111)	C (***)	G (695)	S (43)	H (27)	L (44)	V (25)	E (19)	A (21)				
L (2066)	C (***)	G (255)	S (44)	E (403)	R (209)	V (42)	E (29)	A (28)					
C (***)	G (170)	S (47)	E (261)	R (93)	V (96)	E (92)	A (57)						
E (119)	A (144)	P (331)	K (154)	A (198)		F (69)	F (156)						
Y (1132)	Y (340)	K (76)	A (152)										
T (22)	T (302)												
A (563)	P (180)												
K (87)													

Table 4. (*continued*)

(C) Sequences of the fragments present in the mixture

(1) F–V–N–Q–H–L–C–G–S–H–L–V–E–A	fragment	1–1; approx 150 pmol
(2) V–N–Q–H–L–C–G–S–H–L–V–E–A	fragment	2–14; approx 400 pmol
(3) N–Q–H–L–C–G–S–H–L–V–E–A	fragment	3–14; approx 100 pmol
(4) Q–H–L–C–G–S–H–L–V–E–A	fragment	4–14; approx 150 pmol
(5) H–L–C–G–S–H–L–V–E–A	fragment	5–14; approx 100 pmol
(6) L–C–G–S–H–L–V–E–A	fragment	6–14; approx 100 pmol
(7) C–G–S–H–L–V–E–A	fragment	7–14; approx 200 pmol
(8) E–A	fragment	13–14; approx 150 pmol
(9) L–Y	fragment	15-16; approx 350 pmol
(10) L–V–C–G–E–R–G–F	fragment	17–24; approx 700 pmol
(11) V–C–G–E–R–G–F	fragment	18–24; approx 300 pmol
(12) Y–T–P–K–A	fragment	26–30; approx 350 pmol
(13) T–P–K–A	fragment	27–30; approx 200 pmol
(14) T–P	possible fragment	27–28; not quantifiable
(15) K–A	possible fragment	29–30; not quantifiable
(16) K	possible residue	29; not quantifiable
(17) A	residue	30; approx 600 pmol

Additional free amino acids (first step): Y_{26}, F_{25}, L_{17}, from F_1 to L_6

(D)

F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Peptide sequence:	F	V	N	Q	H	L	C*	G	S	H	L	V	E	A	L	Y	L	V	C*	G	E	R	G	F	F	Y	T	P	K	A.

Table 5. Sequence analysis of the alkaline mixture of procasomorphin (1500 pmol of the original peptide) after 2 hr incubation at pH 8. Peptide sequence is shown at the bottom. (A) Amounts of all PTH-amino acids obtained at each step of the Edman degradation; the data of the identified residues are in **bold**. (B) Residues identified at each step and their amounts data. (C) Sequences of the fragments present in the mixture

(A) Edman steps

	Residues (pmol)																		
	D	N	S	Q	T	G	E	A	H	Y	R	P	M	V	C	W	F	I	K	L
1	8	5	12	2	7	**525**	9	16	—	**512**	7	11	2	**896**	2	14	**461**	**94**	**124**	9
2	7	2	4	2	4	21	4	7	—	31	4	**956**	1	19	1	13	12	7	13	5
3	7	2	3	2	3	11	4	6	—	8	3	45	—	5	—	12	11	**154**	6	4
4	5	2	2	1	3	6	3	4	—	1	2	**116**	—	3	—	11	6	12	2	4
5	5	2	2	1	2	5	3	3	—	1	2	14	—	2	—	10	2	8	56	3
6	4	1	1	1	2	4	3	3	—	—	1	12	—	2	—	10	1	4	7	2

(B) Residues identified at each step

1		2		3		4		5	
V	(896)	P	(956)	I	(154)	P	(116)	K	(56)
Y	(512)								
F	(461)								
G	(525)								
I	(94)								
K	(124)								

(C) Sequences of the fragments present in the mixture

(1) Y–P fragment 2–3; approx 500 pmol
(2) F–P fragment 4–5; approx 500 pmol
(3) G–P–I–P–K fragment 6–10; approx 200 pmol
(6) G–P fragment 6–7; not quantifiable (a value of about 300 pmol can be deduced)
(7) I–P fragment 8–9; approx 100 pmol
(8) K residue 10; approx 150 pmol
Additional free amino acids (first step): V_1 (completely removed)

	1	2	3	4	5	6	7	8	9	10
Peptide sequence:	V	Y	P	F	P	G	P	I	P	K.

dextrose agar, supplemented with 0.5% yeast extract. Preinocula were grown for 5 days at 28° under agitation (180 rpm) into 500 ml flasks with 100 ml of a medium (pH 6.0) containing yeast extract 0.015%, Glc 0.5%, $(NH_4)_2SO_4$ 0.184%, K_2HPO_4 0.177% and KH_2PO_4 0.068%. The mycelium was then ground with an Ultra Turrax mixer TP-18N for 30 sec to break up pellets and to make the inoculum homogeneous. The cultural medium was prepared by adding 6 ml of preinoculum to a 2 l flask containing 750 ml of cultural medium prepared by adding yeast extract 0.5% and Glc 0.2% to the synthetic medium described in Ref. [30]. The culture was grown at 28° under stirring (180 rpm) for 5 days and the broth was clarified by filtration and centrifugation at 12 000 *g* for 20 min. Aliquots of the broth utilized for the incubation mixtures were dialysed against H_2O and immediately used.

Protein concn and tryptic activity. The protein concn was determined using the Bio-Rad Protein Assay kit according to the manufacturer's instructions and using bovine serum albumin as a standard. Tryptic activity was determined by measuring the rate of hydrolysis of *N-p*-toluene sulphonyl-L-arginine-methyl ester as previously described [31].

Peptides digestion. Each peptide (15 nmol) was incubated at 37° with *Trametes trogii* culture broth (proteins broth/peptide ratio 1:2 w/w) in 1.5 ml of either 0.5% NH_4 bicarbonate, pH 8.0, or NH_4OAc 20 mM, pH 5. Aliquots of the incubation mixtures, corresponding to 1500–2000 pmol of the original peptide, were withdrawn at 0, 0.5, 1, 2 and 4 hr, acidified by HOAc and freeze-dried. The freeze-dried samples were then dissolved in H_2O (0.2 ml) and lyophilysed twice.

Sequence analyses. Sequence analyses were performed by using a pulsed liquid-phase sequencer (Perkin–Elmer Applied Biosystems model 477A) equipped on-line with a phenylthiohydantoin amino acid derivatives analyser (Perkin–Elmer Applied Biosystems model 120A). Samples were dissolved in aq. 0.1% TFA (20–30 μl) and loaded onto a TFA-treated glass-fibre filter, coated with polybrene and washed according to the manufacturer's instructions.

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