TRYPSIN AND α-CHYMOTRYPSIN INHIBITORS FROM POTATOES: ISOLATION AND SOME PROPERTIES.

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SUMMARY: At least 13 inhibitors of trypsin and α-chymotrypsin were shown to be present in the juice of potato tubers by polyacrylamide gel electrophoresis. 9 of these proteins were isolated by isoelectric focussing. Isoelectric points, specific activities and amino acid compositions were determined. One of the inhibitors could be shown to exist as a tetramer, which in the presence of α-chymotrypsin dissociates in two steps to form I_2E and IE complexes.

INTRODUCTION: There are many papers dealing with inhibitors for proteolytic enzymes in potato juice. Reviews are given by Vogel et al. (1) and by Liener and Kakade (2). In most instances the inhibitor preparations obtained have not been characterized very well. An exception is the well-studied chymotrypsin inhibitor I, isolated by Balls and Ryan (3). But it also doesn't seen to be a homogeneous protein (4). Recently Hochstrasser et al. (5) isolated 4 substances with inhibitor activity against trypsin and some other proteases. A "...possible existence of multiple forms of the chymotryptic and tryptic inhibitor from Solanum tuberosum..." is postulated by Rola (6,7).

We have detected 13 trypsin and/or α-chymotrypsin inhibitors in the juice of potato tubers by electrophoresis in polyacrylamide gel. 9 inhibitors were isolated by isoelectric focussing and partially characterized.

MATERIALS AND METHODS: Potato tubers ("Maritta") were obtained

from Pfanni-Werk Otto Eckardt KG (München). Proteins of the juice were precipitated at pH 6 with $(NH)_4SO_4$. The precipitate obtained between 0.1 and 0.6 saturation was washed 4 times with acetone/H_2O (2/1, v/v) and 2 times with acetone at 0°C. The yield of air-dried crude inhibitor (RI) was 445 g/50 kg potatoes. 50 g RI were extracted 2 times with 0.01 M CH_3COOH (320 and 180 ml). The combined extracts were lyophilized and yielded 27,5 g TIE_3. - Trypsin (Merck, Nr. 8213) was determined with BAPA using a modified method (cf. Nagel et al., 8). - α-Chymotrypsin (Serva, Nr. 17160) was determined with casein (Merck, Nr. 2242) according to Rick (9). - Polyacrylamide gel electrophoresis was performed according to Bailey (10), using 0.1 M glycine/NaOH buffer pH 9.2. Proteins were stained by amido black 10 B, inhibitors by the method of Uriel and Berges (11). - Isoelectric focussing was carried out according to Haglund (12), using the small column of LKB-Producter AB (LKB 8101). All fractions obtained were analyzed for trypsin and α-chymotrypsin inhibitor activity and controlled by polyacrylamide gel electrophoresis. For isolation of inhibitors ampholines and sucrose were removed by gelfiltration, using BIO-GEL P-2 and P-10. - Amino acid analysis was performed by an Unichrom Analyzer (Beckman). N-terminal groups were identified by the Dansyl-method (13-16). The molecular weights of inhibitor A5 and of its complexes with α-chymotrypsin were estimated by gelfiltration, using Sephadexes G-75 superfine G 200 (17) and by ultracentrifugation (Spinco E, Beckman).

RESULTS AND DISCUSSION: Polyacrylamide gel electrophoresis of TIE_3 at pH 9.2 shows a number of protein bands with activity against trypsin and α-chymotrypsin, migrating towards the

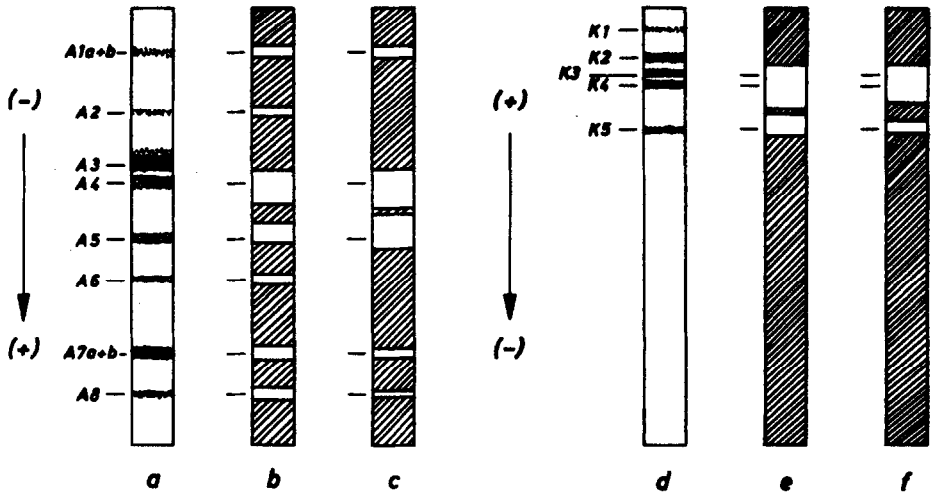

Fig. 1: Polyacrylamide gel electrophoresis of TIE_3, 0.1 M glycine/NaOH pH 9.2, 100 V, 150 min; a,d: protein stain by amido-black. b,e: trypsin inhibitor stain. c,f: α-chymotrypsin inhibitor stain.

cathode and towards the anode (Fig. 1). The anodic bands A9 - A13 have left the gel under the conditions used, but these proteins are without inhibitor activity.

A separation of inhibitors in a micropreparative scale (30 mg TIE_3 per run) was possible by isoelectric focussing in the pH-ranges 5 - 8 and 7 - 10 (Fig. 2a and 3a). In some cases - especially for isolation of inhibitors Ala, A4 and A5 - it was advantageous to start from TIE_3, heated for 3 min. at 95°C in aequous solution: A number of heat-labile inhibitors were destroyed and therefore separation of the remaining heatstabile proteins is improved (Fig. 2b and 3b).

In this way 9 inhibitors were isolated in an electrophoretical pure form. The isoelectric points and the specific activities of these compounds are given in Table 1. As can be seen from Table 2 the amino acid composition also is different.

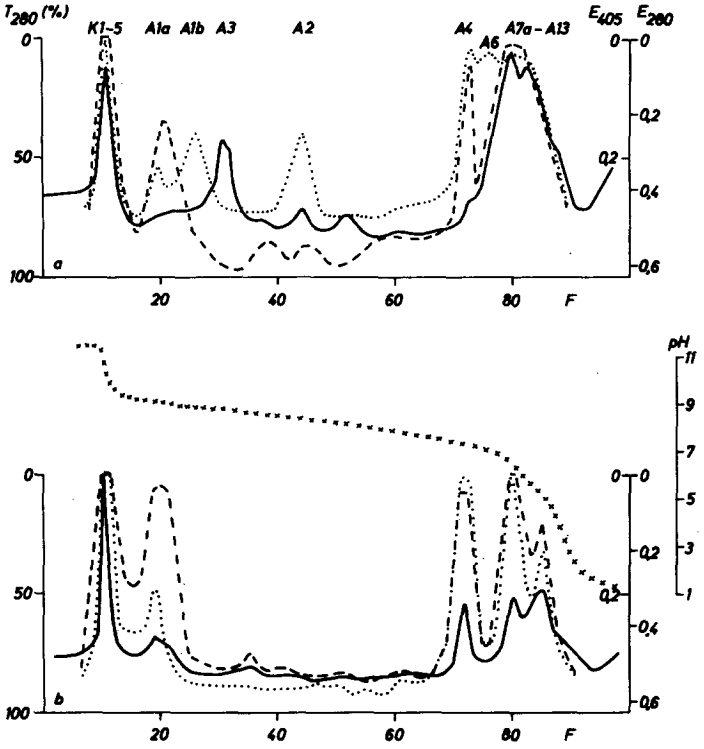

Fig. 2: Isoelectric focussing of a) TIE_3, b) TIE_3 after heating to 95°C for 3 min. pH-range: 7-10, 1 ml-fractions (F). _____ protein (T_{280}), ····· trypsin inhibitor activity (inhibition of 20 µg trypsin by 0.025 ml aliquots, substrate BAPA, E_{405}), ------ α-chymotrypsin inhibitor activity (inhibition of 12 µg α-chymotrypsin by 0.025 ml aliquots, substrate casein, E_{280}), x x x x pH-gradient.

Remarkable is the high cystine content of A4, A5 and A7b - a group of "heat-stabile" inhibitors - in comparison to the low values obtained for the "heat-labile" inhibitors A2, A6 and A8. Complex formation was studied with A5 first of all, because this inhibitor has the highest specific activity against α-chymotrypsin. Dependent on the weight ratio inhibitor/enzyme A5 yields two complexes, which can be separated by electrophoresis in polyacrylamide gel (Fig. 4) and isolated by gel-

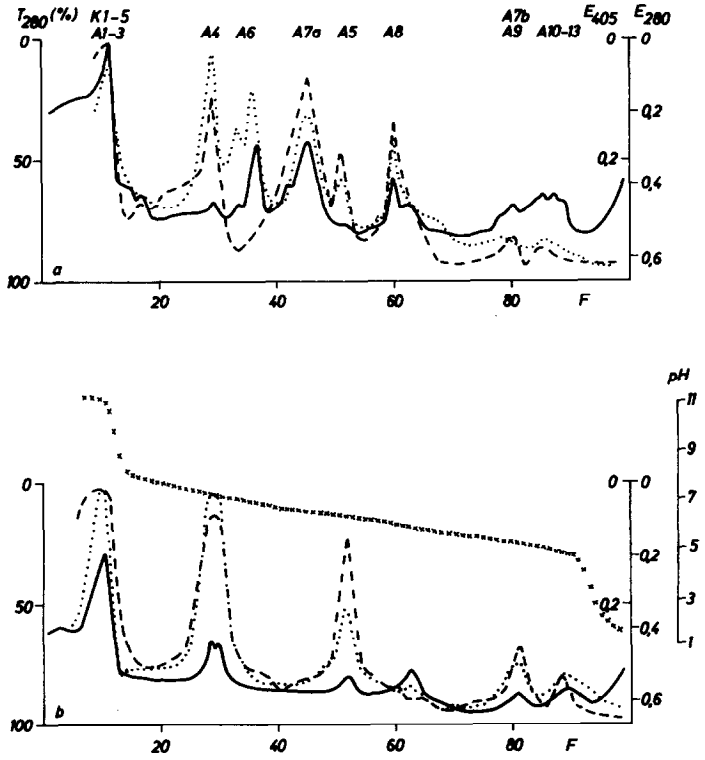

Fig. 3: Isoelectric focussing of a) TIE_3, b) TIE_3 after heating to 95°C for 3 min. pH-range: 5 - 8. For explanation see fig. 2.

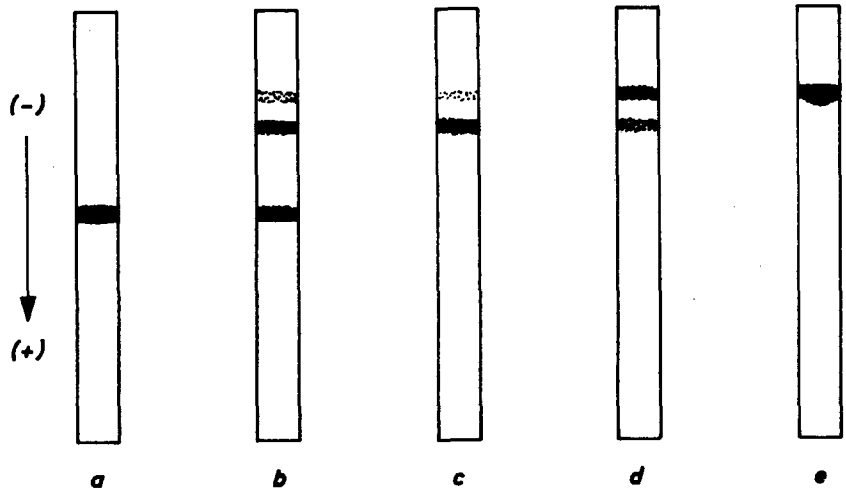

Fig. 4: Polyacrylamide gel electrophoresis of complexes formed by inhibitor A5 and α-chymotrypsin. Conditions see fig. 1, protein stain by amido-black, weight-ratio inhibitor: enzyme a) 1 : 0, b) 1 : 1, c) 1 : 2, d) 1 : 3, e) 1 : 4.

Table 1

Isoelectric points and specific activities against trypsin and α-chymotrypsin

Inhibitor	Isoelectric point (pH)	Inhibition of trypsin[1] (mg E/mg I)	Inhibition of α-chymotrypsin[2] (mg E/mg I)
K4	> 9.2	0.88	0.82
A1a	8.9	0.50	1.15
A1b	8.7	-	-
A2	8.2	1.22	0
A4	7.2	3.54	2.48
A5	6.3	3.45	4.02
A6	6.9	1.28	0
A7a	6.4	0.68	0.54
A7b	5.1	2.25	1.50
A8	5.8	1.22	0.86

[1] Substrate: BAPA; [2] Substrate: casein

filtration. A molecular weight of 22 - 26.000 for A5 was estimated by gelfiltration. Therefore α-chymotrypsin seemed to combine with A5 forming IE_2 and IE_4 complexes. But the molecular weights of isolated complexes were much lower than the values expected for the above formulas. In the presence of 6 M guanidine A5 shows a molecular weight of about 6.000 in the ultracentrifuge (sedimentation - equilibrium - method). This value is in good agreement with the minimal molecular weight (6.400), calculated from the amino acid analysis. This result indicates, that A5 is a tetramer, dissociating in the presence of α-chymotrypsin to form complexes according to the following two-step scheme:

$$I_4 + 2\,E \rightleftharpoons 2\,I_2E \qquad \text{(complex 1)}$$

$$2\,I_2E + 2\,E \rightleftharpoons 4\,IE \qquad \text{(complex 2)}$$

The amino acid composition of the isolated complexes 1 and 2 also confirms this scheme.

Table 2

Amino acid composition

	K4	A4	A5	A7b	A2	A6	A8
	(Mol %)						
Lys	8.9	8.2	7.8	7.7	7.1	6.0	5.6
His	2.5	1.5	0.1	0.0	1.2	1.9	1.3
Arg	4.4	3.8	3.8	4.0	4.2	3.7	3.5
$CySO_3H$[1)]	0.2	1.3	0.3	0.8	0.3	0.5	0.7
Asp	13.4	10.4	11.6	12.5	13.0	12.3	12.9
Thr	4.9	7.7	5.9	6.6	5.0	5.2	6.0
Ser	6.7	7.7	6.9	7.8	7.6	9.2	11.5
Glu	10.7	8.7	9.2	10.3	7.2	7.2	7.5
Pro	6.3	6.2	5.6	6.0	6.7	6.2	4.8
Gly	9.9	12.0	11.4	12.8	10.0	10.2	10.8
Ala	7.3	5.8	5.3	5.4	4.6	4.0	5.1
Cys	3.1	6.0	6.2	5.7	1.3	0.8	0.0
Val	6.0	2.9	1.7	2.2	7.7	7.6	7.4
Met	0.0	0.0	0.0	0.0	0.0	1.4	0.0
Ile	4.1	4.7	4.2	4.6	5.7	5.7	4.7
Leu	5.1	4.3	3.4	3.7	9.3	9.0	9.1
Tyr	2.9	5.8	6.2	6.0	3.7	3.6	3.6
Phe	3.3	2.9	3.8	3.9	5.4	5.6	5.6
Trp	-	-	0.0	-	-	-	-
N-term.			Arg		Arg		Arg

1) The peak appearing at the position of cysteic acid was not identified.

This behavior of A5 is in contrast to that of chymotrypsin inhibitor I of Ryan and Balls (18): CTI I also dissociates into subunits having a molecular weight about 9.000, but this dissociation was observed only in the presence of 3 - 4 M guanidine. In the presence of α-chymotrypsin an IE_4 complex is formed, having a molecular weight above 125.000. Millard et al. (19) and Hochstrasser et al. (20) also observed a dissociation of inhibitors from some plant sources by combining with trypsin.

These results obtained with inhibitor A5 may explain the

appearance of such a large number of inhibitors in potatoes and in other plant materials: Possibly there are only few monomers, which form a large number of oligomers by mixed association.

Acknowledgements

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