Fábián F. - Fülöp L. - Sajgó M.: The Limited Proteolysis as a Potential Tool for Screening and Detection of Special Antinutritive Factors . - In: Bulletin of the Szent István University, ISSN 1586-4502 , 1995. 1995-1996, 87-93. p.

The Limited Proteolysis as a Potential Tool for Screening and Detection of Special Antinutritive Factors

F.Fábián, L.Fülöp, M.Sajgó
Department of Chemistry and Biochemistry, University of Agriculture, Faculty of Agricultural Sciences, Gödöllö, Hungary

Keywords

Saponins, trypsin inhibitors, antinutritive factors, screening

Introduction

The presence of different antinutritive factors in the food- and feedstuffs plays an important role in the production and use of these materials. Some of these compounds have no direct toxic effect, but influences the utilization of other components of the nutrients. The physiological effects and the detailed description of the role of these antinutritive factors are summarized by Liener (1980) and T.Szabo (1982).

The saponins represent an important group of the antinutritive factors. The presence of these compounds can be proved by biologic (Jones, Elliot, 1969; Livingstone et al., 1977; Shany et al., 1970) as well as by chemical methods (Tencer et al., 1972; van Atta et al., 1961; Brawn et al., 1981). Since these compounds are surface active and act as detergents, we tried to find relationship between this character and the antinutritive effect.

The detergents may influence the conformation of proteins by specific and/or the unspecific binding to the protein molecule. This can result in the change of the biological activity or the accessibility of the molecule for proteolytic enzymes. An optimal model for this type of studies was the limited proteolysis of the subfragment 1 (S1) of myosin (Mocz et al., 1984).

A proteolytic enzyme (trypsin) is an essential constituent of the reaction mixture of digesting S1, therefore each component which influence (inhibit) the activity of trypsin can influence the process of the limited digestion of S1 as well. We assumed that the changes in the limited proteolytic digestion in the presence of plant extracts can be due not only to the changes of the conformation of the substrate, but could be attributed to the inhibition of trypsin. This offers a quick method to the screening of the presence of trypsin inhibitors in plants.

Materials and methods

Myosin was prepared from rabbit muscle. The S1 was prepared from myosin, by digestion with chymotrypsin , according to Mócz et al. (1984).

Protein concentration was determined by the absorption at 280 nm, by the use of absorption coefficients ($A^{1\%}_{1cm}$) of 5.6 (myosin) and 7.5 (S1).

The tryptic digestion of S1 was performed in 100 mmol/L Tris-HCl, 5 mmol/L EDTA, at pH 8.0. The concentration of S1 was 1.5 mg/ml, trypsin/S1 ratio was 1/50, digestion was performed for 15 min at 37 °C. Digestion was terminated by the addition of SDS-mercaptoethanol in 1:1 ratio followed by boiling for 5 min. For the digestion TPCK treated trypsin (MERCK) was used.

The different samples tested were dissolved in the buffer used for digestion and added to the digestion mixture. Digestion was started by the addition of trypsin followed by incubation for 10 min at 25 °C.

SDS gel-electrophoresis was performed according to the method of O'Farrell (1975), in acrylamide (12.5%) - bis-acrylamide (0.6%) slab gel, thickness of 2 mm. The amount of protein applied was 20 mg. The slab was stained with 0.1% coomassie brilliant blue dissolved in 50% methanol and 10% acetic acid (O'Farrell, 1975).

For ascending thin layer chromatography MERCK Kieselgel 60 precoated plates were used. Samples were dissolved in 50% ethanol, 100 mg/cm sample was applied. The chromatogram was developed by propanol:ethylacetate:water = 40:40:30 (v/v), at room temperature. The plates were stained with aniline (4% in ethanol): diphenylamine (1%, in ethanol): phosphoric acid = 5:5:1 (v/v) (DAF-reagent), at 90 °C for 10 minutes. (Stahl, 1969). In some cases acetic anhydride:sulphuric acid (98%) = 1:1 (v/v) (AS-reagent) was used (van Atta, Guggolz, 1958)

The samples from the undeveloped section of the plates were scarped into test tubes and extracted twice with acetone. After centrifugation the supernate fraction was evaporated and dissolved in 0.5 ml buffer.

The Carlo Erba saponin was extracted twice by acetone, p-xylole, ethanol, methanol, dimethyl-sulphoxide, hexane and light petrol added in ratio of 1:10 (w/v).

Plant shoots were dried at 90 °C until constant weight, the dry material was minced and extracted twice by acetone or 50% ethanol added in 1:10 (w/v) ratio. The extracts were dried and dissolved in the digestion buffer.

Results and discussion

Effect of commercial saponins on the limited digestion of Subfragment-1

The in vitro tryptic digestion of the subfragment 1 of myosin is a limited process, under special conditions (Mocz et al., 1984). Since the limited character of the digestion is attributed to the native structure of the protein, all the conditions which affect this structure can influence the character of proteolysis as well.

We tested a set of commercial saponins (ICN, Reanal, Sigma, Fisher, Carlo Erba, Koch-Light, BDH., Merck, Eastman) for this denaturation effect.

Since by gel electrophoresis is easy to follow the character of the digestion, we used this technique (Fig.1.). According to the

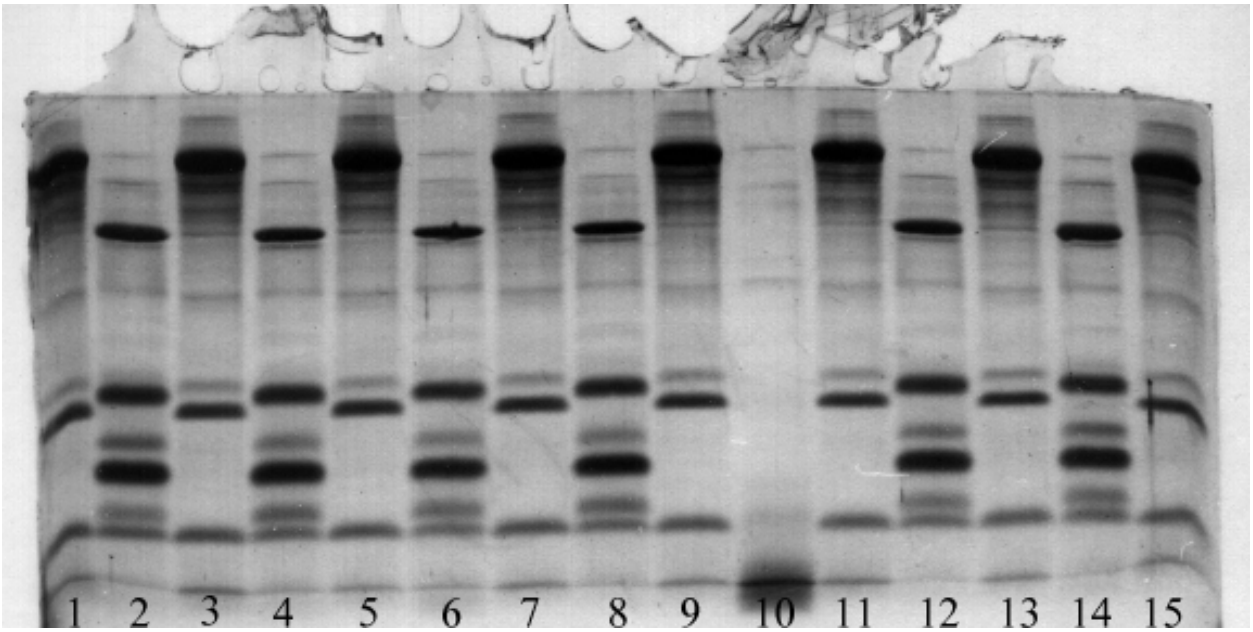

Fig. 1. The limited proteolysis of S1 in the presence of different commercial saponins. The saponin: S1 ratio was 1:2 (w/w). The control S1 is in the slots (1,3,5,7,9,11,13,15); in the slots (2,4,6,8,10,12,14) the tryptic digestion of the S1 in the absence of added saponin, in the presence of commercial saponins from ICN, Reanal, Sigma, Carlo Erba, Koch-Light and Fischer, respectively.

gel electrophoresis pattern only the Carlo Erba product changed the result of tryptic digestion (Fig.1., sample 10). In the presence of this saponin the limited character of the digestion was suspended, the digestion process did not stop, the subfragment 1 was completely digested.

This result shows that the saponins generally do not affect the steric structure of S1 to that extent which should result in the change of the limited character of proteolysis. The only exception out of the samples tested was the saponin produced by the Carlo-Erba.

The partial purification of the denaturing agent (DA) from the Carlo Erba saponin

The Carlo Erba product is not homogenous, therefore we tried to isolate the active component, which has direct role in the denaturation. The components of the saponins were separated by preparative thin-layer chromatography (Fig.2). The

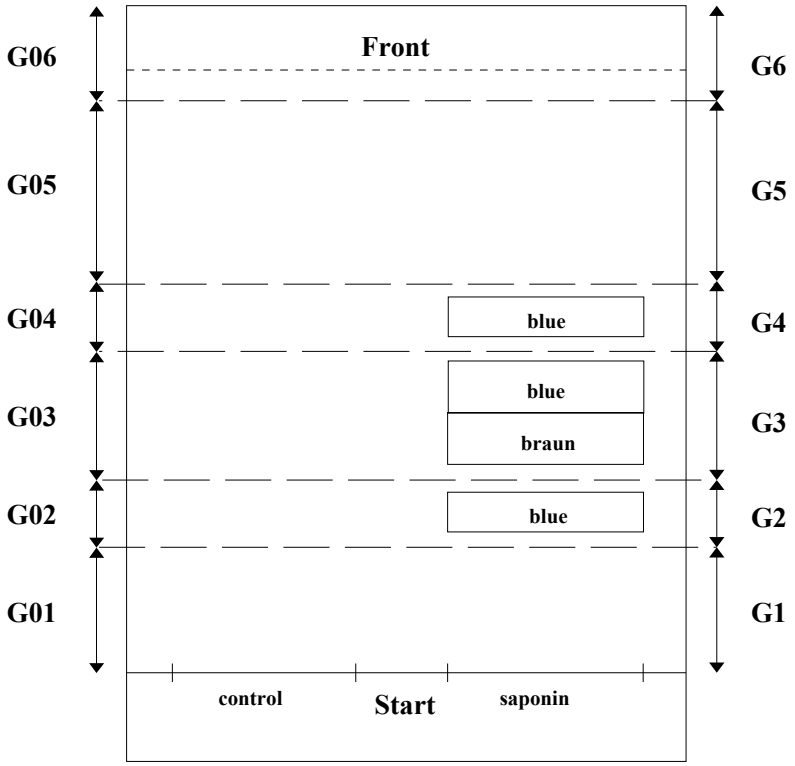

Fig.2. Isolation of the Carlo-Erba saponin fractions by TLC. The control bands were stained with DAF reagent. The bands of the control zone (G01-G06) and the saponin containing zone (G1-G6) were eluted. The other details are described in Matherials and Methods.

marked bands were eluted and separately tested for denaturing effect, by gel electrophoresis (Fig.3). According to the denaturation test, only fraction G5 out of the six fractions was positive.

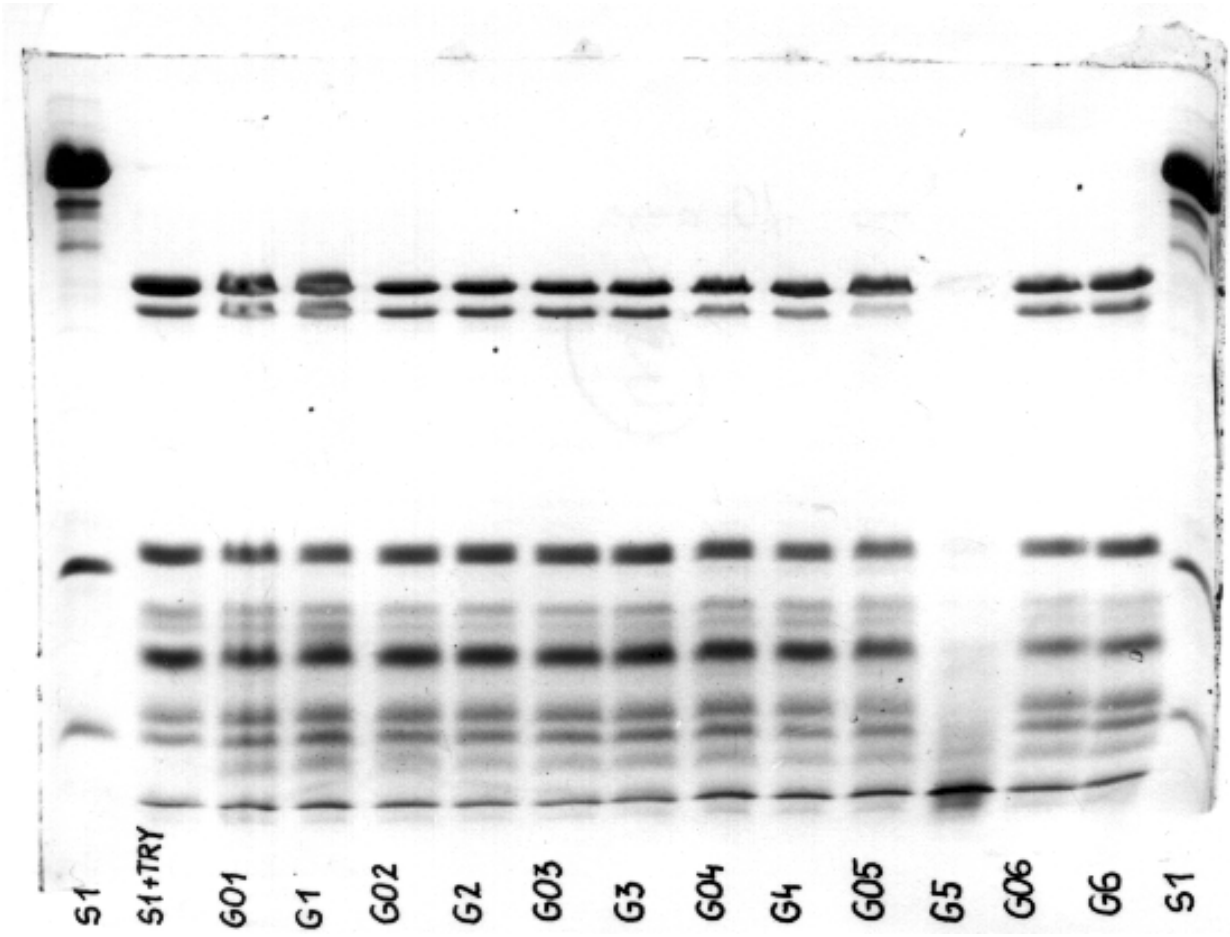

Fig. 3. The effect of the TLC-fractions on the limited proteolysis of S1. The fractions studied were eluted as it is described on Fig.2., (S1) undigested S1, (S1/TRY) S1 digested in the absence of added fractions. The other abbreviations are the same as in Fig.2.

A separate part of the plate containing fractions from G1 to G6 was stained for saponins. Surprisingly, the G5 area of the plate did not give any color reaction characteristic to saponins, not even with the most sensitive iodine-test.
The quantitative determination of the active component (DA) was not possible by gravimetry, since the elute of plate contained a significant amount of inactive components - as proved by the elution of the control bands. However, the denaturing effect of the DA was ten times as effective as the original Carlo-Erba sample.

The direct extraction of the Carlo-Erba product by different organic solvents (see Matherials and methods) and the test of the extract proved that by acetone and ethyl-acetate the extraction was successful (Fig. 4), but did not result in the

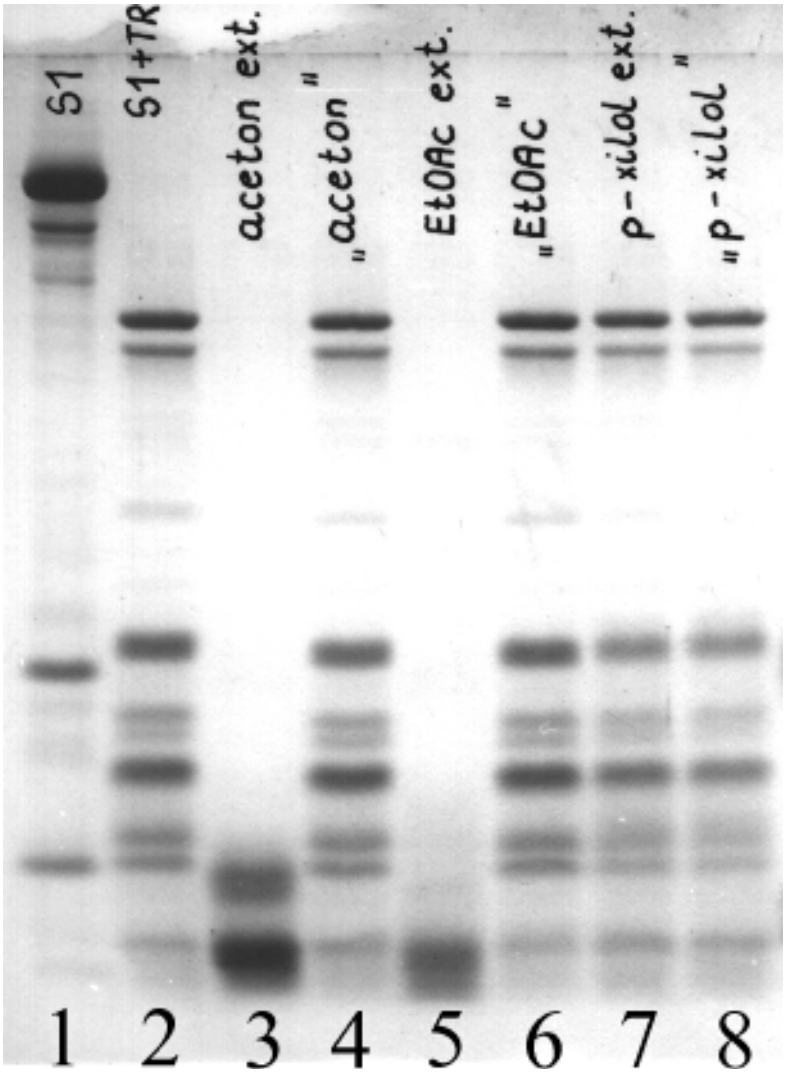

Fig.4. The effect of different extracts of the Carlo Erba saponin on the limited proteolysis of S1. (1) control S1, (2) the tryptic digestion of control S1, (3) acetone extract, (4) the residue of the pure acetone, (5) ethylacetate extract, (6) residue of the pure ethylacetate, (7) p-xylole extract, (8) residue of the pure p-xylol.

homogeneity of DA, since all of these extracts also contained saponin positive components , tested by thin layer chromatography. The solvents used for extraction did not contain DA-active component as it is shown on Fig. 4.

Based on these results we assume that this digestion test is not suitable to the detection of saponins, but called our attention to and other denaturing component soluble in acetone which can be the constituent of materials of plant origin.

Detection of the trypsin-inhibitory compounds by limited proteolysis

Since trypsin an essential component of the limited proteolysis test, all the factors that influence trypsin activity can affect the process of the proteolysis.
It is well known that in lucerne out of the saponins different type of trypsin-inhibitors are present as antinutritive factors (Liener, 1980, Green and Ryan, 1972). To eliminate the effect of the DA component, we extracted the air-dry shoots of high and low-saponin lucerne with 50 per cent ethanol. In this solvent the DA component is not soluble. The extracts depending of their origin more or less changed the progress of digestion (Fig.5.) The extract of the lucerne of low saponin content changed the

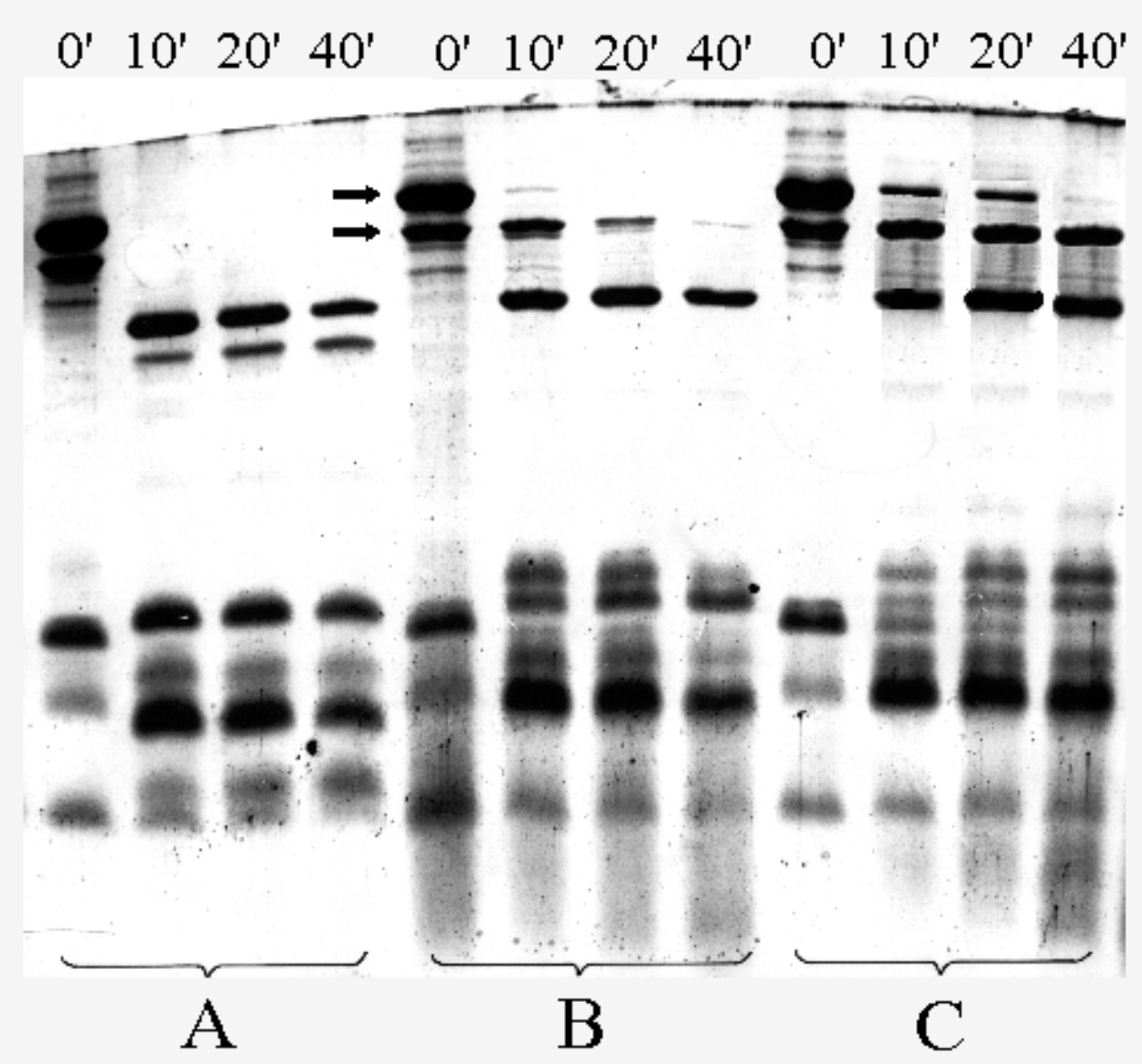

Fig. 5. The effect of the high and low saponin containing lucerne extracts on the limited proteolysis of the myosin S1. The time course of the limited proteolysis in the absence of extract (A), in the presence of the low (B) and high (C) saponin containing lucerne extracts. The extracts added represents 0.03 grams of air dried lucerne.

electrophoretic pattern (Fig.5B) by decreasing of the digestion rate, the disappearance of the S1 fragments marked by arrow was much slower as in the control (Fig.5A). This effect was more characteristic in the case of the extract coming from the high saponin containing lucerne (Fig.5C). This is in a good agreement with the experience that lucerne of high saponin content has a higher trypsin inhibitor activity.

We assume that these differences in the rate of tryptic digestion is related to the trypsin inhibitor content. We should like to emphasise that this inhibitory effect of the plant extract (TG activity) can be due to other than the trypsin inhibitor of protein type.

To prove the method in practice we examined 23 plants of 15 families. Ten of the plants tested is important as feed- or foodstuff (Table 1.). The air-dried

Table 1. The trypsin inhibitor (TG), denaturing agent (DA) and saponin (S) content of 23 plant species.

TG	DA	S	TG+DA	TG+S	DA+S	TG+DA+S	X
3	1	2	0	5	5	2	5

(X) the number of the uncertain or partial results. Other details are described in Materials and Methods and in the text.

plants were extracted by acetone for DA and 50 per cent ethanol for trypsin inhibitor. The presence of saponin was given by literature (Rapoti and Romvari, 1983). There is not a straight correlation between the saponin content, DA and TG activity. However, TG and/or DA occurs in plants containing saponin (12 of the 16 tested).

Summary

A model system is presented for the screening of plant materials for trypsin inhibitor (TG) and for an antinutrient denaturing factor (DA). The basis of this test is the affecting of the limited tryptic digestion of the subfragment 1 (S1) of myosin. The limited character of the proteolysis of S1 is suspended in the presence of the DA, and the rate of the proteolysis is decreased in the presence of inhibitor. For extraction 50 per cent ethanol (TG) and acetone (DA) was used. This method is suitable for screening tests, since the gel electrophoresis

monitoring system is suitable for screening of 20 samples per day, by the use of a slab gel electrophoresis. The screening of 23 plant species for DA and TG proves that in plants the DA and TG together are not common, it occurs mostly in the presence of saponin.

References

Brawn, P. R., Lindner, N. M., Miller, J. M. and Telling, G., M. 1981. A gas chromatographic method for the determination of medicagenic acid in lucerne (Alfalfa) leaf protein concentrate. J. Sci. Food Agric. 32: 1157-1162.

Green, T. R. and Ryan,, C. A. 1972. Wound-induced proiteinase inhibitor in plant leaves. A possible defense mechanism against insects. Science 175: 776-777

Jones, M. and Elliott, F. C. 1969. Two rapid assays for saponin in individual Alfalfa plants. Crop. Sci. 9: 688-691.

Liener, I. E. 1980. Toxic constituents of plant foodstuffs. I.,E. Liener ed. Second Edition, Academic Press,

Livingston, A. L., Whitehand, L. C. and Kohler, G. O. 1977. Microbiological Assay for saponin in Alfalfa products. J. of A.O.A.C. 60: 956-960.

Mocz, G., Szilagyi, L., Lu, R.C., Fabian, F., Balint, M. and Gergely, J. 1984. Effect of nucleotides, divalent cations and temperature on the tryptic susceptibility of myosin subfragment 1. Eur. J. Biochem. 145:221-229.

O'Farrel 1975. High resolution two-dimensional gel electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Rapoti, J. and Romvary, V. 1983. Gyógyító növények. J. Rapoti and V. Romvary, Eds. Medicina, Budapest

Shany, S., Birk, Y., Gestetner, B. and Bondi, A. 1970. Preparation, characterisation and some properties of saponins from lucerne tops and roots. J.Sci.Fd Agric. 21: 131-135.

Stahl, E. 1969. Thin-layer chromatography. Stahl, E., Ed. Second Ed.:856. Springer-Toppan.

Tencer, Y., Shany, S., Gestetner, B., Birk, Y. and Bondi, A. 1972. Titrimetric method for determination of medicagenic acid. J. Agr. Food Chem. 20: 1149-1151.

T.Szabo, M. 1982. Növényi eredetû tápanyagok biológiai értékét csökkekntô antinutritív tényezôk. Állattenyésztés és takarmányozás 31: 273-287.

van Atta, G. R. and Guggolz, J. 1958. Detection of saponins and sapogenins on paper chromatograms by Lieberma-Buchard reagent. Agric.Food Chem. 6:849-850.

van Atta, G. R., Guggolz, J. and Thopson, C. R. 1961. Determination of saponins in Alfalfa. Agr. Fd Chem. 9: 77-80.