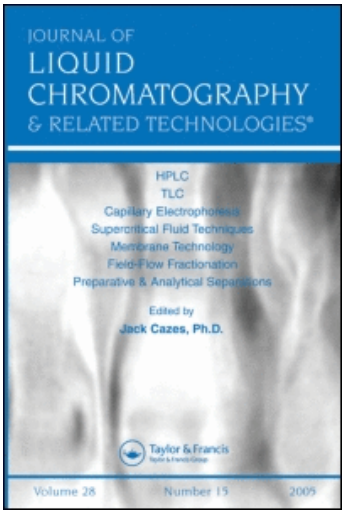

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Characterization of Proteintype Proteinase Inhibitors by High Performance Capillary Electrophoresis

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CHARACTERIZATION OF PROTEINTYPE PROTEINASE INHIBITORS BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method based on high performance capillary electrophoresis (HPCE) has been developed for identification and quantitative determination of inhibitors from leguminous seeds. The method allows specific characterization of Kunitz soybean trypsin inhibitor (KSTI) and Bowman Birk inhibitor (BBI) from soybean (*Glycine max.*, L.). Standard curves for the inhibitors showed a satisfactory linearity between normalized peak area and sample concentration, and a good repeatability of the method was found. Identification of protein peaks as proteinase inhibitors was based on their binding to trypsin. The system also proved useful for determination of the number and specificity of inhibitor sites on

the individual inhibitors and for binding studies with monoclonal antibodies against the inhibitors.

INTRODUCTION

Proteintype proteinase inhibitors for both trypsin and chymotrypsin are widespread in plants.[1] The inhibition of these digestive enzymes are expected to account for some of the antinutritional problems observed when inhibitors are present in food and feed and especially soybean constituents have been the subject for various investigations in this connection.[2,3]

The inhibitors present in soybeans are divided into two groups of isoinhibitors, the Bowman Birk inhibitors (BBI) and the Kunitz soybean trypsin inhibitors (KSTI).[1] BBI isoinhibitors form a group of approximately eight doubleheaded inhibitors with molecular weights of 8 kD and high cystine contents.[4] The KSTI represents a group of three isoinhibitors with molecular weights of 20.1 kD and two disulfide bridges.[5]

As a wide variety of isoinhibitors with different properties often occurs in soybeans as well as in other plant varieties[1], measurements of the inhibitor activity by enzymatic assays does not always provide sufficient informations.[3] The evaluation of the significance of a certain level of inhibitor activity[6] may also require information on the number and relative amounts of the individual inhibitors and their specificities, number of inhibitor sites and affinities for enzymes.[7]

For rapid evaluation of the inhibitor contents in large numbers of samples, techniques based on monoclonal antibodies in enzyme linked immunosorbent assays (ELISA) have proven useful.[8-10] As basis for these types of assays and for various other reasons it is also important to obtain knowledge on the specificity of the mAb and if possible the epitope to which the mAb binds on the inhibitor. The ELISA offers the possibilities for such analyses.[11,12] However, the ELISA involves hydrophobic interaction of the first layer with the plastic surface of the microtiter wells used for ELISA. This hydrophobic interaction has been suspected to cause partly denaturation of the protein[13] and in addition the molecular interaction does not necessarily occur at the same rate in solution as when one of the components has been subjected to immobilization. There is, therefore, a need for supplementary methods for efficient characterization of the inhibitors. The use of HPCE offers a technique with the possibility of studying complex formation without immobilization or

labelling of the reactant and still employing buffer conditions suitable for the binding reactions to occur.[14]

Previously, we have developed an HPCE method for separation of proteintype inhibitors of proteinases and their complexes with monoclonal antibodies and enzymes.[7] This HPCE method used MECC with cholate micelles and taurine in the buffer or mobile phase. The cholate micelles give both hydrophobic and ionic interactions with the proteins whereas the applied zwitterion, taurine, protects against adsorption of proteins to the capillary wall.[7,14]

The present paper describes uses of MECC with a buffer system and pH suitable for detailed characterization of proteintype proteinase inhibitors. The binding between these inhibitors and the enzymes, trypsin and chymotrypsin, as well as the monoclonal antibodies against the inhibitors are studied. The technique allows specific determination of proteins which have binding sites for the enzymes and epitopes corresponding to paratopes on the antibodies.

MATERIALS

Apparatus

The apparatus used was an HP^{3D}CE (Hewlett-Packard, Waldbronn, Germany) with a 614 mm x 0.05 mm I.D. fused-silica capillary. Detection was performed by on-column measurements at a position 530 mm from the injection end of the capillary.

Samples and Reagents

Bowman Birk inhibitor (BBI) from soybean, Kunitz soybean trypsin inhibitor (Type I-S) (KSTI), porcine pancreas trypsin and bovine pancreas chymotrypsin were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies raised against the inhibitors from soybean were from the collection in this laboratory.[12] Trigonellinamide, disodium hydrogenphosphate, cholic acid and taurine (2-amino-ethanesulphonic acid) were obtained from Sigma.

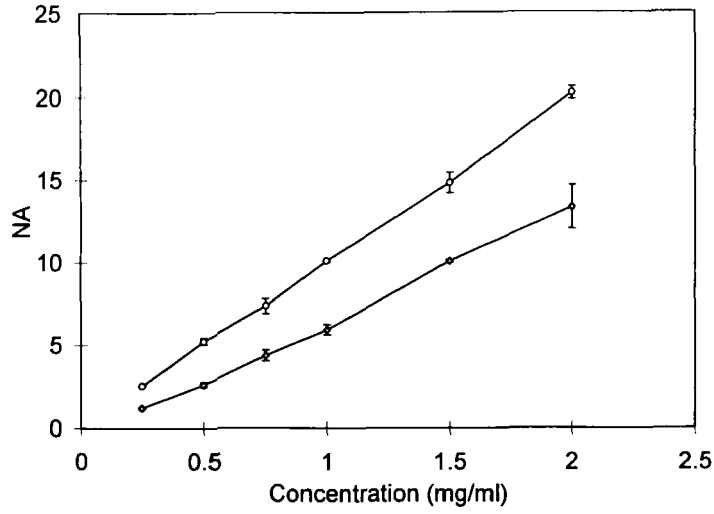

Figure 1. Normalized areas (NA) measured in triplicate from electropherograms of varying amounts of BBI ( ) and KSTI (O).

METHODS

All analyses with enzymes were made with solutions of enzymes in running buffer freshly prepared from standard solutions of enzymes in 1 mM HCl. The samples were introduced from the positive end of the capillary by vacuum injection (2 sec., 25 mbar). On-column detection at 200 nm was applied. Buffer solutions were filtered through a 0.2 μm membrane filter before use. Washing with 0.1 M NaOH for 2 min and buffer for 5 min were performed between each analysis.

RESULTS

The applicability of HPCE for quantitative determination of inhibitors was evaluated by the linearity obtained when normalized peak areas were plotted against sample concentration. Triplicate runs were performed at 6 different concentrations of the inhibitors, BBI and KSTI (Fig. 1). Different slopes were found due to different response factors of the inhibitors at 200 nm.

The linearity studies also included trypsin, which is a proteinase inhibited by BBI and KSTI, α-lactalbumin as a reference protein/inert protein and a marker molecule; trigonellin amide. The data obtained are displayed in Table 1 and the presented relative standard deviations for the NA determinations are given as the highest of the six relative standard deviations obtained. For all components tested, good correlation was found between normalized peak areas and sample concentration (Table 1). Similarly, the variation in migration time was examined. Peak identification by migration time was seen to be somewhat enhanced by use of migration times relative to the internal markers. In addition, the use of an internal marker provides a reference for alignment of electropherograms of different compounds.

Table 1

Correlation Between Normalized Peak Area (NA) and Sample Concentration as well as Relative Standard Deviation (Rel Std Dev, Calculated from n Runs) for Migration Time (MT) and Relative Migration Time (RMT)

Sample	Conc. range (mg/ml)	NA=f(C)* Corr. coeff.	NA rel std dev/(n) (%)	MT rel std dev/(n) (%)	RMT** rel std dev/(n) (%)
BBI	0.25-2.0	0.9979	<9.9 /(4)	0.58/(24)	0.53/(24)
α-lactalbumin	0.25-2.0	0.9936	<11.8/(3)	0.99/(18)	0.63/(18)
KSTI	0.25-2.0	1.0000	<6.5 /(4)	1.02/(24)	1.07/(20)
Trypsin	0.25-2.0	0.9960	<5.5 /(3)	2.84/(18)	2.06/(18)
TGA	0.06-0.5	1.0000	<7.5 /(4)	0.66/(20)	

* C; Concentration of sample

**RMT; MT relative to MT of TGA (trigonellin amide) used as internal standard

The designation of particular protein peaks to inhibitors was investigated by the ability of the protein to form complexes with the proteinase enzymes, hence resulting in appearance of a complex peak with migration time different from the individual components. All mixed samples were allowed to form complexes in 5 mM phosphate buffer at pH 7.5, and MECC of the

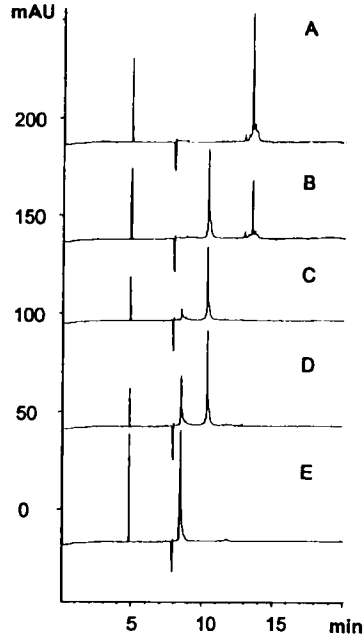

Figure 2. Complex formation of KSTI and trypsin at different mixture ratios of A: KSTI (2.4 mg/ml) + TGA (0.6 mg/ml). Electropherogram E shows porcine trypsin (2.73 mg/ml) + TGA (0.27 mg/ml). For B, C and D the molar ratios of KSTI to trypsin was 1.6, 0.8 and 0.4 respectively.

mixtures were performed immediately. Fig. 2 shows results from the mixing of KSTI and trypsin at different molar ratios followed by electrophoresis. KSTI is seen to have one inhibitor site for trypsin as the NA of the complex peak did not increase regardless of whether the enzyme or the inhibitor is present in a molar excess. Self digestion of trypsin was investigated by repeated runs of a KSTI-trypsin mixture at pH 8.2, which did not show degradation of the complex peak (Fig. 2, C) during more than 3 hours.

In Fig. 3, the binding between KSTI and a monoclonal antibody against KSTI is shown at various molar mixing ratios, with the mAb-KSTI complex having a migration time different from those of the individual molecules. As expected, a molar excess above 2 of KSTI to mAb was necessary to observe a peak for unbound KSTI, implying that the mAb bound two KSTI molecules per mAb molecule (Fig. 3, C).

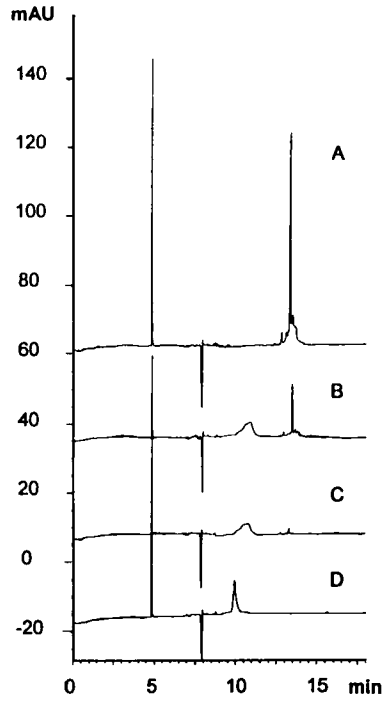

Figure 3. Complex formation of KSTI and a monoclonal antibody with specificity for KSTI. Electropherogram A (KSTI (2 mg/ml) + TGA (1 mg/ml)) shows the mixture used for incubation with the mAb. B: KSTI:mAb in molar ratio of 3.6:1; C: KSTI:mAb in molar ratio 1.8:1; D: mAb (1.07 mg/ml) + TGA (0.6 mg/ml).

The ability of KSTI to simultaneously bind trypsin and the mAb was investigated by mixing the mAb-KSTI complex from Fig. 3 with trypsin at different molar ratios (Fig. 4). The inhibitor site for KSTI was seen to be different from the mAb binding site as simultaneous binding was observed.

The chymotrypsin-trypsin inhibitor, BBI, was subjected to similar studies, with incubation of BBI with chymotrypsin at different molar ratios (Fig. 5). Similarly to the KSTI-complexes, the formation of complex peaks resulted in changed MT's.

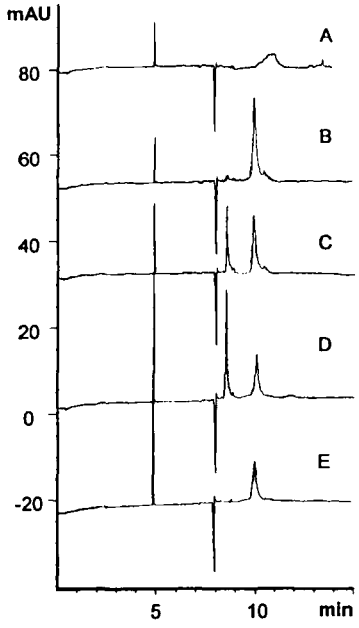

Figure 4. Simultaneous binding of mAb and trypsin to KSTI. A: Mixture of mAb and KSTI in a molar ratio of 1:1.8. The KSTI + TGA mixture employed in A, B and C are identical to Fig. 3, A. B: KSTI:mAb:trypsin in a molar ratio of 1:0.56:1.5; C: KSTI:mAb:trypsin in a molar ratio of 1:0.56:3; D: mAb (0.84 mg/ml) + trypsin (0.5 mg/ml) + TGA (0.5 mg/ml); E: mAb (1.07 mg/ml) + TGA (0.6 mg/ml).

DISCUSSION

HPCE was investigated for its applicability as a method of characterization of proteintype proteinase inhibitors. The linear correlation shown between NA and the concentrations of the analytes (Table 1) enables the use of NA as a measure of the amount of individual proteins. As the response factors differ for the individual proteins (Fig. 1), an evaluation of the amounts of proteins in the complex peaks from NA requires knowledge of the response factor of the complexes. Instead, an internal marker, TGA, has been included in the inhibitor samples used for mixing with other proteins. The NA of TGA

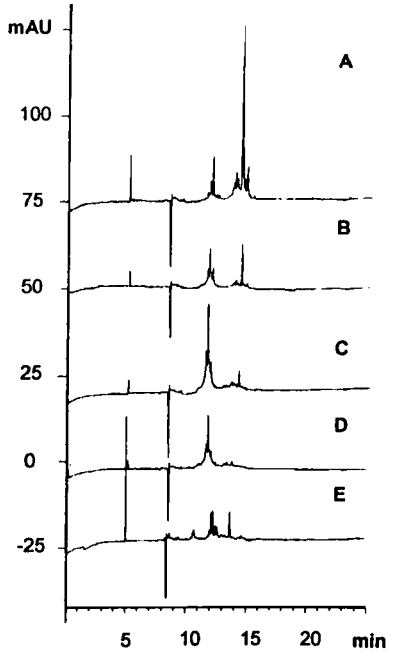

Figure 5. Complex formation of BBI with chymotrypsin. A: Shows the mixture used for incubation with chymotrypsin; BBI (2.73 mg/ml) + TGA (0.1 mg/ml); B: BBI:chymotrypsin in a molar ratio of 2.7:1; C: BBI:chymotrypsin in molar ratio 1.4:1; D: BBI:chymotrypsin in a molar ratio 0.8:1; E: chymotrypsin (2.4 mg/ml) + TGA (0.2 mg/ml).

was also shown to be linearly correlated with the concentration (Table 1), and the amount of TGA observed in an electropherogram with inhibitor present can therefore be used as a measure for the total amount of the inhibitor present in the sample. The use of an internal marker may provide a better determination of the mixing ratios than when calculated from the microlitres actually mixed due to the errors correlated to pipetting of volumes of a few microlitres.

In Fig. 2, the complex formation between KSTI and porcine trypsin is illustrated. High affinities are usually observed for the binding of inhibitors to trypsin and chymotrypsin and the reaction rate is high resulting in fast complex formation following mixing of the compounds.[6,7] With mixing of bovine chymotrypsin and KSTI no complex formation was observed, confirming the findings that KSTI does not have an inhibitor site for chymotrypsin[15] although

this has been indicated in some studies.[16] These results also indicate that the system now developed does not favour unspecific complexation.

When mixing KSTI with mAb, a broad peak was observed (Fig. 3). This peak broadening may be due to a mixture of mAb complexed with one or two KSTI molecules. With addition of trypsin to the mAb-KSTI mixture, a new complex peak was formed with markedly changed MT and peak shape (Fig. 4, B and C). In a sample with trypsin and mAb, no interaction of the trypsin and mAb was observed (Fig. 4, D and E), confirming that trypsin binds to KSTI in the KSTI-mAb complex.

With binding of BBI to chymotrypsin, a range of inhibitor-enzyme complex peaks appeared, which possibly is due to the presence of various isoforms of BBI as well as of chymotrypsin.

In conclusion, MECC offers a method requiring limited sample amounts compared to traditional methods and with complex formation in solution which complements traditional methods as ELISA and enzymatic assays. With the ability of HPCE to be used for quantitative analyses of the proteins, the method presented enables knowledge of the number and specificity of inhibitor sites on the individual isoinhibitors present in a given sample as well as characterization of mAb binding to the inhibitors.

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REFERENCES

1. M. Richardson, "Seed Storage Proteins: The Enzyme Inhibitors," in **Methods in Plant Biochemistry**, L. J. Rogers, ed., Academic Press Ltd., London, 1991, vol. 5, pp. 259-305.

2. M. L. Kakade, D. E. Hoffa, I. E. Liener, J. Nutr., **103**, 1772-1778 (1973).

3. B. Bjerg, B. O. Eggum, H. Sorensen, in **Recent Advances of Research in**

Antinutritional Factors in Legume Seeds, Pudoc, Wageningen, The Netherlands, 1989, pp. 351-358.

4. A. L. Tan-Wilson, J. C. Chen, M. C. Duggan, C. Chapman, R. S. Obach, K. A. Wilson, J. Agric. Food Chem., **35**, 974-981 (1987).

5. J. H. Orf, T. Hymowitz, Crop. Sci., **19**, 107-109 (1979).

6. A. M. Arentoft, K. Mortensen, H. Sørensen, Aspects of Appl. Biol., **27** 211-215 (1991).

7. A. M. Arentoft, H. Frøkiær, S. Michaelsen, H. Sørensen, S. Sørensen, J. Chrom. A, **652**, 189-198 (1993).

8. D. L. Brandon, A. H. Bates, M. Friedman, J. Food Sci., **53**, 102-106 (1988).

9. D. L. Brandon, A. H. Bates, M. Friedman, J. Agric. Food Chem., **37**, 1192-1196 (1989).

10. A. M. Arentoft, H. Frøkiær, H. Sørensen, S. Sørensen, Acta Agric. Scand., Sect. B, Soil and Plant Sci., **44**, 236-243 (1994).

11. D. L. Brandon, A. H. Bates, J. Agric. Food Chem., **36**, 1336-1341 (1988).

12. H. Frøkiær, L. Hørlyck, V. Barkholt, H. Sørensen, S. Sørensen, Food & Agric. Immun., **6**, 63-72 (1994).

13. S. E. Dierks, J. E. Butler, H. B. Richerson, Mol. Immun., **23**, 403-411 (1986).

14. S. Michaelsen, H. Sørensen, Polish J. Food Nutr. Sci., **3**, 5-44 (1994).

15. H.-D. Belitz, J. K. P. Weder, Food Rev. Int., **6**, 151-211 (1990).

16. J. K. P. Weder, Adv. Exp. Med. Biol., **199**, 239-279 (1986).

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