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Direct determination of kallikrein by high-performance liquid chromatography

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ABSTRACT

A direct and specific identification of porcine pancreatic kallikrein by high-performance hydrophobic chromatography is proposed; the minimum amount which can be injected is 2.5 U. An application to the quantitative determination of the enzyme by high-performance size-exclusion chromatography is reported; the method is precise with a mean coefficient of variation of 2.8% and the minimum amount which can be injected is 0.02 U of kallikrein. The results obtained with determinations in real biological samples (porcine pancreatic powder and human urine) are reported. The method is based on direct and specific chromatographic signals and does not destroy the biological activity of this enzyme.

INTRODUCTION

Kallikrein is a serine protease occurring in various tissues and body fluids of humans and other mammals. Various methods [1–17] have been reported for the determination of kallikrein activity. Measurement by a synthetic peptide substrate assay using esters [6–12], *e.g.* N^{α}-benzoyl-L-arginine ethyl ester (BAEE) [6], is commonly used; however, these substrates are not specific for kallikrein, but show a generic esterase activity.

This paper proposes a direct and specific identification of porcine pancreatic kallikrein by high-performance hydrophobic interaction chromatography (HPHIC) and an application to the quantitative determination of this enzyme by high-performance size-exclusion chromatography (HPSEC) in various batches of commercial products. The determination of the enzyme is accomplished without the use of a substrate and possible errors owing to the presence of compounds with esterase activity are avoided.

This is a preliminary contribution to the study of the behaviour of mammalian kallikreins in high-performance liquid chromatography (HPLC) owing to the limited number of reports [13–16] describing the use of HPLC for this analysis.

EXPERIMENTAL

Reagents and materials

All chemicals were of analytical-reagent grade or the highest purity available and were stored, when necessary, as recommended by the manufacturer. HPLC-grade ammonium sulphate was from Bio-Rad Labs. (Richmond, CA, USA); porcine pancreatic kallikrein was from several lots from Sigma (St. Louis, MO, USA), Calbiochem-Behring Diagnostics (Scoppito L'Aquila, Italy) and Unibios (Trecate Novara, Italy); aprotinin was from Sigma. Water was distilled once and then deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Aprotinin was bound to cyanogen bromide (CNBr)-activated Sepharose 4B according to the method given by the manufacturer (Pharmacia, Uppsala, Sweden); prolonged washing cycles of alternately high and low pH were necessary to obtain an acceptable HPLC background.

Econo columns, 10 × 1.0 cm I.D. (Bio-Rad), were used for the affinity chromatography.

Columns

HPHIC was performed on a 75 × 7.5 mm I.D. column of Bio-Gel TSK Phenyl-5-PW (Bio-Rad). HPSEC was performed on a 300 × 7.5 mm I.D.

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Ultropac column of TSK G3000 SW (LKB-Pharmacia).

Apparatus

An LKB-Pharmacia DfB HPLC System One, equipped with a Rheodyne 7125 injector, a 100-μl injection loop and a LC 2249 gradient pump was connected to a variable-wavelength 2141 monitor. The UV detector was operated at 220 nm. The output from the detector was displayed on the 2221 integrator (LKB-Pharmacia).

Chromatographic separations

HPHIC was carried out at a flow-rate of 1.0 ml/min with a 30-min linear salt gradient obtained controlling buffer A (1.8 *M* ammonium sulphate in 0.1 *M* phosphate, pH 7.0) and buffer B (0.1 *M* phosphate, pH 7.0) using a solvent programmer at 20–25°C.

HPSEC was performed using a mobile phase of 0.15 *M* phosphate buffer in 0.1 *M* sodium chloride at a flow-rate of 1.0 ml/min. Bovine serum albumin (MW 67 000), ovalbumin (45 000), α-chymotrypsinogen (25 000) and cytochrome C (12 400) were used as standards for the calculation of molecular weight. A linear calibration graph of retention time *versus* log molecular weight is shown in Fig. 1.

Inhibition assays

Inhibition assays were performed using BAEE as a substrate according to the method of Trautschold and Werle [6]. A Perkin-Elmer Lambda 17 spectrophotometer was used for absorbance measurements.

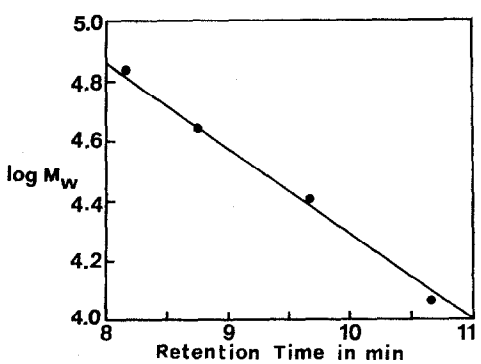

Fig. 1. Calibration graph obtained using protein molecular weight standards.

Procedure

Aqueous solutions of porcine pancreatic kallikrein, buffered with 0.1 *M* phosphate (pH 8.0) containing *ca.* 100 U of enzyme, were prepared and 100 μl, containing about 10 U were directly assayed by the HPHIC technique.

Samples prepared as above were transferred onto the aprotinin–Sepharose 4B column (bed volume *ca.* 2 ml) previously equilibrated with 0.1 *M* sodium hydrogencarbonate in 0.5 *M* sodium chloride (pH 8.3). The eluate was passed through the same column twice. The column was then washed with at least ten bed volumes of the same buffer solution to remove unbound substances prior to elution. The kallikrein bound to immobilized aprotinin was fully recovered by elution with 0.1 *M* sodium acetate–acetic acid (pH 4.5) in 1.0 *M* sodium chloride. At this pH value, the enzymatic activity does not decrease. The resulting solution, *ca.* 80 ml, was concentrated by ultrafiltration through an Amicon YM-10 membrane to 5.0 ml, and a 100-μl aliquot was assayed by the HPHIC technique.

Amounts of kallikrein in the 1–10 U range were processed through the aprotinin column as described above and were recovered in 5.0 ml. A 100-μl volume of the resulting solution was assayed by HPSEC.

Calibration graph

Aliquots of porcine pancreatic kallikrein resulting from the second procedure were standardized by the BAEE method and analysed by HPSEC as calibration samples. The calibration graph of peak area *versus* units of kallikrein injected (0.02–20) was obtained from a least-squares linear regression.

RESULTS AND DISCUSSION

Preliminary investigations carried out with reversed-phase (RP) HPLC columns were unsuccessful, despite the different hydrophobicities of the phases used. With the HPHIC column good results were obtained in terms of resolution, the reproducibility of peak areas and recovery. Fig. 2 shows the chromatogram resulting from the direct injection of an aliquot of a commercial sample. Extraneous substances, inactive *versus* aprotinin, can be removed by processing, as described under Experimental.

Fig. 3 shows the elution profile obtained; it is typ-

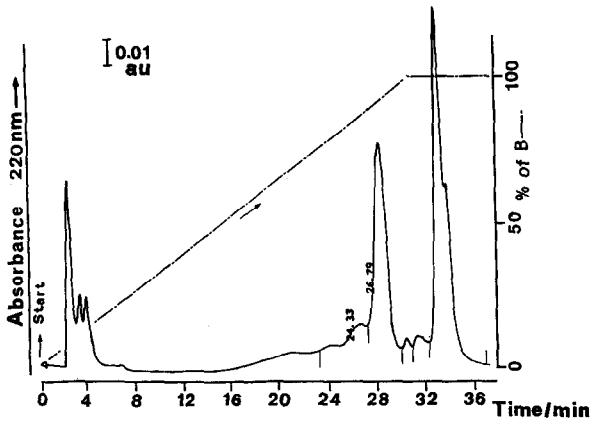

Fig. 2. Chromatogram of commercial pure porcine pancreatic kallikrein (2.5 U in 0.1 ml) obtained on Bio-Gel TSK Phenyl 5PW column (75 × 7.5 mm) at a flow-rate of 1.0 ml/min with a 30-min linear gradient of ammonium sulphate concentration from 1.8 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0). Percentage of B indicates the percentage of buffer B.

ical for all batches of porcine pancreatic kallikrein (Sigma and Calbiochem) examined and is characterized by the appearance of peaks a–d. According to other workers [3], multiple forms are observed for this enzyme, explained in terms of a intra-chain split occurring in the various purification steps. The volume fraction corresponding to the elution profile of the various peaks of kallikrein (Fig. 3) was examined for enzymatic activity. As a reference, an amount of kallikrein equal to that injected was diluted to the same volume fraction with the same chromatographic eluent mixture; the esterase activity shown by the chromatographic effluent and by the reference solution was almost identical, indicating a quantitative recovery from the HPLC column. The minimum injectable amount which could be determined by the HPHIC technique was 2.5 U.

Among the active substances which can be expected in samples containing kallikrein is trypsin, although this enzyme can be selectively inhibited by the addition of soybean trypsin inhibitor [3]. Eluate fractions corresponding to the single peaks a–d (Fig. 3) were collected and separately injected for measurement by HPSEC. All the forms gave the peak shown in Fig. 4. The molecular weight of this compound was calculated to be *ca.* 30 000, compatible with that of porcine pancreatic kallikrein [3]. These results show that the multiple peaks observed in the elution profile obtained with HPHIC (Fig. 3) are due to forms of kallikrein with similar structures.

The response obtained with HPSEC has been used for the quantitative determination of kallikrein in the diluted samples, owing to its higher sensitivity than HPHIC. The results are reported in Table I. The minimum amount in the original samples which can be detected is 1.0 U. The minimum amount which can be injected is 0.02 U.

These results are promising enough to justify de-

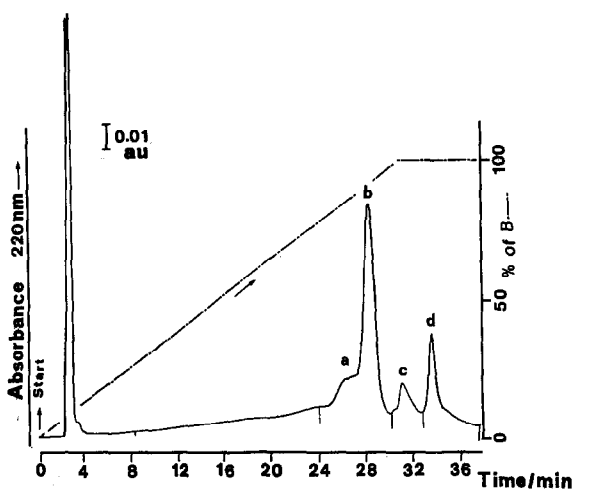

Fig. 3. Chromatogram of processed pure porcine pancreatic kallikrein (2.5 U in 0.1 ml). Conditions as in Fig. 2.

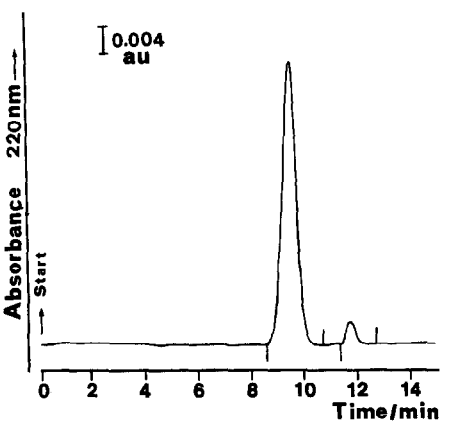

Fig. 4. Chromatogram of processed pure porcine pancreatic kallikrein obtained on TSK G3000 SW column (300 × 7.5 mm) using a mobile phase of 0.15 *M* phosphate buffer (pH 7.0) in 0.1 *M* sodium chloride at a flow-rate of 1.0 ml/min.

TABLE I

EVALUATION OF THE ACCURACY, PRECISION AND RECOVERY OF THE METHOD

Mean coefficient of variation is 2.8%.

Sample No.	Amount of kallikrein processed[a] (U)	Amount of kallikrein found[b](U)	C.V. (%)	Recovery[c] (%)
1	1.00	0.96 ± 0.043	4.5	96
2	2.00	1.94 ± 0.04	2.6	97
3	5.00	4.90 ± 0.11	2.2	98
4	8.00	7.60 ± 0.19	2.5	95
5	10.0	9.90 ± 0.22	2.2	99

[a] Standardized by BAEE method.
[b] Mean ± S.D. ($n = 5$).
[c] Calculated from the activity measured in the samples collected after chromatographic elution.

termining the chromatographic behaviour of kallikrein in real biological samples such as crude kallikrein from procine pancreatic powder (Unibios) and human urine.

Crude kallikrein

Porcine pancreatic powder was suspended in buffer solution B and filtered through a Minisart NML Sartorius 0.2-μm filter. Figs. 5 and 6 show the chromatograms obtained before and after purification with aprotinin according to the first and second procedures. In Fig. 5 the elution profile of kallikrein is hidden by several peaks of extraneous substances present in the raw material. Only peak d is predominant in Fig. 6 and the elution profile is similar to that showed in Fig. 3; the predominance of only peak d can be explained by the small number of purification steps undergone by the raw material.

Human urine

The pH of a measured volume of urine (1500 ml) from a normal subject was adjusted to pH 8.0 by the addition of 2 *M* sodium hydroxide. After centrifugation (4390 *g*) for 10 min, the supernatant was transferred by a peristaltic pump (P1, Pharmacia, flow-rate 5.0 ml/min) to the aprotinin–Sepharose

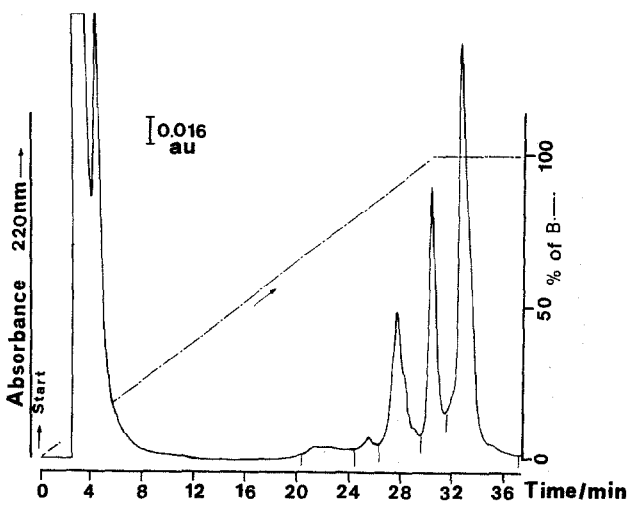

Fig. 5. Chromatogram obtained from porcine pancreatic powder dissolved in buffer B, filtered through a Minisart NML Sartorius 0.2-μm filter and directly injected onto the column. Conditions as in Fig. 2.

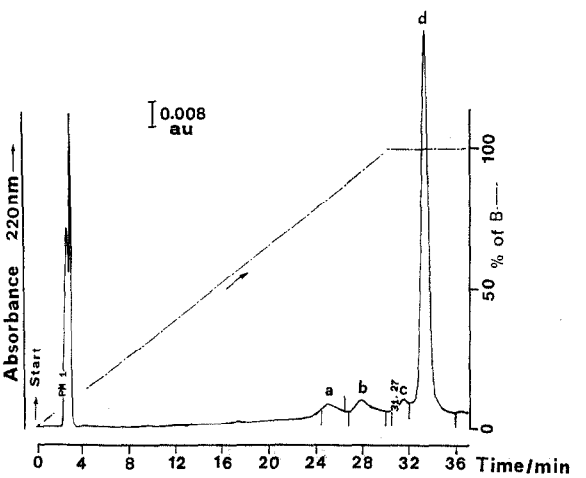

Fig. 6. Chromatogram of processed kallikrein from the crude material of Fig. 5. Conditions as in Fig. 2.

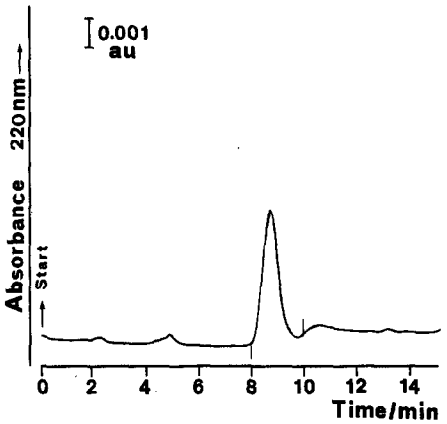

Fig. 7. Chromatogram of processed human urinary kallikrein. Conditions as in Fig. 4.

4B column. The affinity column was washed with about 200 ml of equilibrating buffer (0.1 *M* sodium hydrogencarbonate in 0.5 *M* sodium chloride, pH 8.3) before the elution of urinary kallikrein. The enzyme was eluted with 0.1 *M* acetate buffer, pH 4.5, and collected in 5.0 ml of buffers. The very low amount of urinary kallikrein recovered does not allow a signal to be obtained with the HPHIC column.

Fig. 7 shows the elution profile obtained by injecting 100 μl of sample onto the HPSEC column. The molecular weight was calculated to be *ca.* 45 000, according to the reported value [16,17].

Work is now in progress to establish a precise, specific and convenient assay for urinary kallikrein.

CONCLUSIONS

From the data presented here, it can be seen that HPHIC and HPSEC are suitable methods for the identification and determination of kallikrein. The procedures provide adequate alternatives to the sometimes complicated separation techniques and the unspecific determination currently used. The methods show an adequate accuracy, which is indicated by the almost complete recovery of kallikrein over the concentration range studied, and does not destroy the biological activity of this substance. The method could be used as a control in the production of kallikrein; the method is advantageous because it is based on direct and specific chromatographic signals.

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