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Review

Kallikrein and kallikrein-like proteinases: purification and determination by chromatographic and electrophoretic methods

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Abstract

Kallikreins and kallikrein-like enzymes make up a family of serine proteinases present in tissues and body fluids of mammals and in some snake venoms. This review deals with the procedures of purification, detection and determination of these enzymes by chromatographic and electrophoretic methods. The procedures are reported in tables, described and discussed with the aim of illustrating the state-of-the-art of research in the field.

Keywords: Reviews; Enzymes; Kallikrein; Kallikrein-like proteinases; Proteinases

Contents

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1. List of abbreviations

BA	Benzamidine
HF	Hageman factor
HF_a	Activated HF
HK	High-molecular-mass kininogen
IgG	Immunoglobulin G
LK	Low-molecular-mass kininogen
PABA	*p*-Aminobenzamidine
RIA	Radioimmunoassay
mRNA	Messenger RNA
SBTI	Soybean trypsin inhibitor
SMG	Submandibular glands

2. Introduction

The term kallikrein is often used generically to encompass a family of serine proteinases with specific and limited proteolytic actions. They are found in various mammalian organs; by means of enzymic action, kallikreins release vasoactive peptides (kinins) from endogenous substrates (kininogens) and for this reason kallikreins are also called kininogenases, or kininogenins, a generic name which includes other enzymes such as trypsin, plasmin, and snake venom proteinases.

From a historical point of view, in the twenties Frey and co-workers [1,2] found that human urine contains a thermolabile and non-dialysable substance having hypotensive properties. A few years later, Werle and co-workers [3,4], having found a similar substance in the pancreas of men and various animals in such great amounts that the pancreas could be taken for its site of origin, named it kallikrein, by derivation from the Greek word $\kappa\alpha\lambda\lambda\iota\kappa\rho\epsilon\alpha\varsigma$, or pancreas. Since further discoveries of similar enzymes in plasma, salivary glands and kidney, many more kallikrein-like enzymes have been found in cells and biological fluids of mammals and other species. It is now clear that the different kallikreins are serine proteinases with a significant chemical similarity, to be classified as a family, and that, like other enzymes, they occur in multiple forms [5–8].

Kallikreins are glycoproteins; they can be divided into two distinct groups [9,10], plasma and tissue kallikreins. The two types of enzymes have received different Enzyme Commission (EC) numbers. They differ in their molecular mass and p*I* values (Table 1), in their amino acid sequences, in immunological characteristics and, to a certain extent, in the profiles of their enzyme activity. Plasma kallikreins play an important role in the pathway of blood coagulation and fibrinolysis [11–13]. Tissue kallikreins, although expressed primarily in the pancreas, have been found in the brain, stomach, adrenal gland, prostate [5,9], small and large intestine, salivary glands, kidney and urine [14], and also in plasma or serum [15]. True tissue kallikreins are classically defined by their ability to generate vasoactive kinin peptides from kininogen [9]. Kallikreins are present at very low levels of concentration both in organs and in biological fluids: by way of example, some reported contents of both the types of enzymes in man and rat are listed in Table 2.

Various aspects of the biochemistry, physiology and molecular biology of kallikreins have been reviewed [11,16–24]; however, this constitutes a rapidly expanding area of interest and an increasing quantity of information is becoming available with regard to the identification of potential, new family members. In the last decade more than 2000 reports have been published. In order to codify family member designations, correct redundancies and clarify ambiguities, a nomenclature for tissue kallikreins has been recently proposed [25].

Table 1
Some reported physicochemical properties of kallikreins and kallikrein-like enzymes

Source	Enzyme[a]	p*I*	M_r (SDS-PAGE)		Gel permeation	Ref.
			non red.	red. (h.c.)		
Human plasma	prekallikrein	8.6–9.5	88; 85	88; 85		[26–28]
	I and II		82; 80	82; 80		[29]
	kall.[b]		88; 85	(53)		[28]
	glandular kall.				40	[30]
Human urine	prokallikrein			48	53	[31]
	latent kall.				55	[32]
	kallikrein	3.75–4.25		(41)		[33]
	kall. A, B and D	3.80; 4.15 4.20			23; 27; 25	[34]
Human pancreas	kallikrein		52	(52)	48	[35]
Human SMG	kallikrein		42		30	[36]
Human salivary gland	prokallikrein	4.6		35		[37]
Human saliva	kallikrein	4.2		33	28	[36]
Porcine pancreas	α-kall. B′	4.32; 4.19		36		[38]
	β-kall. A	4.19[c]	25	(17.1)	28.3	[39–42]
				(16.3)		[42]
	β-kall. B	4.21[c]	30.3	(20.8)	32	[39,40] [42,43]
				(19.0)		[42]
	β-kall. C			(17.1)		[41]
Porcine urine	kall. A and B			36.1; 39.6		[44]
Porcine SMG	kall. A and B	3.15–4.4		35.9; 39.6		[44]
Mouse SMG	mGK6		27; 17	(27)		[45]
	Kall-A		24			[46]
	Kall-B		27	(17)		
Rat plasma	kallikrein		91.3[d]	41.3[d]	180[e]	[47]
Rat stomach	kallikrein	4.05			29	[48]
Rat heart	kallikrein			38		[49]
Rat SMG	rK1	3.8–4	27	38		[50,51]
		3.50–3.85	25.4–28.2	31.4		[52]
	rK2	6.2	25	35		[53]
		6.05–6.15	25.7	29.2		[54]
	rK7	5.2	27	(19.0)		[50,51]
		5.15	25.1	(17.2)		[52,55]
	rK8	5.1	27	(18.0)		[51]
	rK9	6.85	25	(20)		[53]
		6.75–6.95	25.8	(16.5)		[56]
	rK10	4.3	27.5; 26.5 26	21		[57,58]
		3.95–4.25	28.4	(19)		[59]
	kall.-like T-kininogenase	4.65–4.75	28	(22)		[60]
Rat pancreas	prokall. A	4.25	36.5	36.5	35.9	[61]
	kall.[f]		34.0	34.0		[62]
	prokall. B	4.35	38.4	38.4	37.4	
	kall.[f]		35.5	35.5		
Rat prostate	rK9 isoenzymes					
	pKLP2-9	6.75–8.35	24.6–29.3	16.5–29.3		[52]
	pKLP2			(16.5)		
	pKLP3			(16.6)		

(*Continued on p. 268*)

Table 1 (*continued*)

Source	Enzyme[a]	p*I*	M_r (SDS-PAGE)		Gel permeation	Ref.
			non red.	red. (h.c.)		
Rat spleen	kallikrein			38		[63]
Rat adrenal glands	kallikrein				33	[64]
Rat urine	kallikrein	4.0	38	38	38	[65]
				43		[66]
	inactive kall.				44	[67]
Guinea pig plasma	prekallikrein	9.0	92	82	99	[68]
	kall.[g]		92	(52)		
Guinea pig SMG	kallikrein		29	29	34	[69]
Bovine pancreas	prokall. A	4.55	26.9	26.9	25	[70]
	kallikrein A			27	25	[71]
	prokall. B				26.7	[72]
	kallikrein B	4.05–4.35		26		
Dog pancreas	CPK-A		33	(16)	36	[73]
	CPK-B		27	(16)	33	
	kallikrein			38		[74]
Rabbit urine			30			[75]
Agkistrodon P. piscivorus venom	kall.-like	7.8		29		[76]
B. arietans venom	kall.-like	4.4		58		[77]
A.h. ussuriensis venom	kall.-like	3.7		24		[78]
L. muta venom	kininogenin	5.4		29.7		[79]
V.a. aspis venom	kall.- like	4.1		43		[80]

Abbreviations: h.c. = heavy chain; p*I* = isoelectric point; M_r = relative molecular mass.

[a] According to the Authors' designations.

[b] Obtained by HF_a activation of the proenzyme.

[c] Asialo-forms.

[d] Labelled with ^{125}I-DTyr-LGlu-LPhe-LLys-LArg chloromethyl ketone.

[e] Homodimer.

[f] Produced by trypsin activation of the zymogen.

[g] Obtained by a prekallikrein activator from skin.

2.1. Some biochemical and physicochemical aspects of kallikreins

The kallikreins of all mammalian plasmas occur prevalently in the form of an inactive precursor (zymogen), prekallikrein, which is bound to high-molecular-mass kininogen (HK). In most tissues and body fluids, kallikreins exist both in a preactive and in an active form in varying proportions. Enzymic activation of the inactive form may take place either spontaneously (autolysis) or purposely, through the action of serine proteinases (e.g., trypsin, Hageman factor (HF), or possibly kallikrein itself) [87,88].

Kallikreins act with the utmost celerity on native kininogen to produce kinins: plasma kallikrein releases bradykinin mainly from HK, unlike from tissue kallikrein (except rat enzyme), which usually splits mammalian low-molecular-mass kininogen (LK) and HK, releasing kallidin. Under normal conditions, the elaborate system of reaction mechanisms, known as the kallikrein–kinin system, is well regulated by inhibitors that limit the concentrations

Table 2
Some reported kallikrein concentration ranges in tissues and body fluids

Source	Concentration		Ref.
	ng/ml	ng/mg	
Human blood plasma	34 500		[81]
Human prostatic secretion	50–2779		[82]
Human saliva	1141–6116		[83]
Human seminal plasma	13–201		[83]
Human serum	0.2–2.2		[83]
Human sweat	0.12–8.4		[84]
Human urine	195–558		[83]
Rat adrenal glands		30.7±4.8[a]	[64]
Rat heart		3.5±0.05[b]	[49]
Rat kidney		9.6±1.7[b]	[85]
Rat pancreas		970[b]	[61]
Rat SMG		220 000[b]	[86]
Rat spleen		0.31–0.35[b]	[63]
Rat urine	ca. 10 000		[65]
Rat blood plasma	55 000		[47]

[a] mg of protein after gel permeation chromatography.
[b] mg of protein in homogenate.

of the active components [89–95]. Progress in the study of kallikrein inhibitors has enabled further development of the purification procedures of kallikreins by affinity adsorption methods [96–105]. Circulating kininogen, however, is not the only substrate for kallikreins: this family of enzymes are trypsin-like in amino-acid sequence and activity, but possess a much narrower range of substrate specificity than trypsin. Kallikreins are capable of hydrolyzing synthetic N-terminally-protected arginine esters (for this reason they are often referred to as arginyl esteropeptidases), and this ability of theirs has been used and extended to an ever-increasing list of new substrates to determine the activity of kallikreins by chemical assay [106–113].

When several kallikreins coexist in a single tissue, serious problems arise for their identification and characterization. The carbohydrate content of tissue kallikrein gives the molecule a heterogeneous electrophoretic mobility reflected also by the broadness of the bands observed in disc or sodium dodecyl sulphate (SDS) electrophoresis and by the presence of numerous forms with different isoelectric points [40,114–123].

Because of the number of kallikreins and kallikrein-like enzymes, it is only possible to trace a broad outline of their pH stability: from pH 4–6 to 10–12, with optimum activity around pH 7–8. Therefore, during the chemical procedures in which the biological activity of kallikreins must be conserved, care must be taken not to subject the enzymes to the potentially denaturing condition of a low pH. Also on their thermal stability it is difficult to generalize: a solution (pH 6–8) of pig urinary kallikrein was stable under refrigeration for several days [114]. Kallikrein-like enzyme from *C. ruber ruber* venom was stable up to 60°C and retained considerable activity (70%) at 80°C [124,125]. Human urinary kallikrein kept at −30°C was stable for more than 12 months; after storage at 4°C for more than 6 months in the presence of NaN_3 (0.02%) the enzyme did not show any appreciable loss of activity, and at 20°C (pH 7. 0) for more than 14 days [126].

3. Purification procedures for preparative or characterization purposes

Since the works of Frey and co-workers, much progress has been made toward the isolation of kallikreins in pure and biologically active forms; the older methods tend to be nonspecific by present-day standards and thus not very efficient, and to cause denaturation and hence loss of activity. In this chapter greater attention will be paid to the reports describing procedures which have been proposed in the last fifteen years. These procedures are essentially chromatographic and electrophoretic.

The newer purification methods may vary according to the chosen purposes (preparation of pharmaceuticals or standards [127] for the determination of enzymatic activity or for structure analysis), and usually employ mild conditions of pH, temperature, and solvent, thus favoring a better retention of biological activity. Operations are frequently carried out at low temperatures (e.g. at +4°C) to hamper micro-organism growth, minimize enzyme–substrate reaction and prevent degradation of thermolabile forms.

Moderate values of pH and ionic strength, as well as a careful choice of organic solvent, limit the possibility that denaturing conditions may arise. In the purification procedures of an inactive zymogen, prolonged steps during which considerable autolysis is likely to occur must be avoided, because in these

cases the final preparations are likely to contain modified forms of the native enzyme [114]. Therefore,it is advisable to work as fast as possible in the first steps in order to minimize the premature activation of the enzyme. When operating with plasma samples for prekallikrein purification, the use of plastic or siliconized glass containers throughout all chromatographic steps is emphasized [27,28,68,128] to minimized contact activation of HF.

The possible addition of preservatives (NaN_3), stabilizing factors ($CaCl_2$), reversible inhibitors [soybean trypsin inhibitor (SBTI), hexadimethrine bromide (Polybrene)], or non-ionic detergents (Triton X-100, Brij 35 [polyoxyethylene(23)lauryl ether]) is suggested [28,31,129,130] both to protect and to activate the substances sought, and to limit interference.

However, it must be borne in mind that during the various stages of purification a more or less sensible decrease in overall activity is unavoidable, so that one must aim to obtain an enzyme pure enough for the intended purpose, reducing the cost/performance ratio to a minimum [131].

The purification of kallikreins can be summarised in three main stages: (1) sample preparation and enrichment procedures for obtaining the concentrated extract; (2) purification of the enzyme(s); (3) characterisation of the kallikrein(s) isolated.

3.1. Sample preparation

3.1.1. Sample collection and storage

Various operations must be performed on the raw material in order to prepare an extract containing the kallikrein in a soluble form. Sample collection may constitute an essential step, mainly when pro-forms are sought, in view of the presence in tissues of interfering components which have not been carefully excluded. Thus, in the purification of latent kallikrein from human urine, sample collection from female subjects eliminated the presence of kallikrein-related enzymes and other proteinases of male accessory sex glands [32]; in the purification of prokallikrein from rat pancreatic tissue, samples were taken without any intestinal tissue, because even traces of the latter produced an active enzyme on simple homogenization [61].

An outline of the preliminary treatments applied to the more common body fluids and tissues follows. If human blood is the raw material [128], this can be collected and mixed (6:1) with acid–citrate–dextrose anticoagulant [citric acid (1.36%), sodium citrate (2.5%) and dextrose (2%)]; the plasma obtained by centrifugation (3000 *g*; 20 min at 22°C) is recentrifuged (5000 *g*; 40 min at 22°C) and kept frozen (−70°C) until use.

Urine from animals is collected [65] into plastic tubes, filtered through several layers of cheesecloth and stored at −20°C; if from humans, it is held on ice during the collection period, stored at 4°C until chilled, and then centrifuged (20 000 *g*; 30 min) to remove precipitated salts and proteins [32].

The tissue (porcine pancreas), stored on ice, is freed from fat and connective tissue, minced and homogenized with 0.1 *M* acetic acid containing SBTI (0.03%) and EDTA (0.1%); the homogenate is centrifuged, the supernatant is decanted and mixed with phosphate buffer solution adjusting the pH value to 7.0. The precipitate is rehomogenized, centrifuged and the second supernatant, brought to pH 7.0, is combined with the first one [114].

As can be deduced from the last example, after membrane disruption the endocellular kallikreins are dissolved and homogenized in a buffer. Various chemical (oxycholate, EDTA) or physical methods (freezing and thawing phases, solid shear) allow or aid membrane disruption. However, a considerable part of the enzyme(s) may remain adsorbed within the cellular membranes and rehomogenization is frequently used.

Crude snake venoms are currently available as lyophilized powder which is dissolved at a concentration of ca. 20% in 0.01 *M* Tris–HCl buffer (pH 8.0), 0.01 *M* in NaCl and any insoluble material is removed by centrifugation (2000 *g*; 30 min at 4°C) [76,77].

3.1.2. Concentration techniques

Owing to the generally low concentrations of kallikrein present both in body fluids and in tissues (Table 2), in many cases large amounts of material have to be worked up. An extreme example is furnished by the purification of kallikrein from human urine: it is evident that in order to provide even a few mg of pure enzyme, some hundred liters of this urine must be processed. Furthermore, work-

ing with solutions having higher concentrations of the enzyme limits its loss by non-specific adsorptions, helps handling, and improves efficiency in subsequent steps of purification (e.g. affinity). At this stage, methods of high capacity rather than resolution are chosen; in practice, besides dialysis and ultrafiltration, precipitation and adsorption techniques are frequently used. Sometimes these preliminary operations conclude with freeze-drying of the concentrated extract.

Ultrafiltration, chiefly used when enrichment is more important than the yield of the protein, is reported as the first preconcentration step especially for urine [31,32,66,75], or as the only step following the centrifugation of a water suspension of hog pancreas powder [132]; membrane and/or hollow-fibre apparatuses (typical molecular mass cut-offs of 10 000, tangential flow) are used [31]. Dialysis is a widespread method, advisable for salt removal or buffer change between two purification steps: it appears as the only step in the treatment of plasma or various homogenates [45,49,63,64,68]; at times as a preliminary step, repeated if necessary, in urine processing [133]; usually as a step following ultrafiltration and precipitation [31,65,75,134,135]. Dialysis is carried out overnight, usually at 4°C, against Tris- or phosphate buffer (10–50 m*M*) [32,48,64], or simply distilled water [35,73].

The precipitation technique based on the salting-out of proteins, is likely to be used directly on the starting medium after removal of insoluble contaminants. Ammonium sulphate is the most commonly used salt because it is cheap and sufficiently soluble; fractionate precipitation (30~75% saturation) is frequently used in urine and homogenate treatments, in order to obtain a higher degree of purification [35,65,114,134]. The liability of the salt to be carried with the precipitate needs a following desalting step such as dialysis. In some instances, the addition of sodium chloride to a concentration of ca. 0.6 *M* has been made during urine processing to separate other components by precipitation (e.g., Tamm-Horsfall protein): after centrifugation (3000 *g*; 30 min), the supernatant was dialysed [31].

An alternative to precipitation is selective adsorption of kallikrein on a appropriate support, for example on an ion-exchange resin. This may be a useful batchwise operation because in controlled pH conditions the technique can be exploited for its high separation rather than preconcentration properties. Thus, homogenates resulting from human [35], bovine [70–72] or dog pancreas [73,74] as well as from human [36] or guinea pig [69] submandibular glands (SMG) are stirred, for a time varying from one case to another, with DEAE-Sephadex A-50 or DEAE-Cellulose, the mixture is packed in a column, washed with acetate or Tris buffer, pH 6.7–8.0, and then eluted by using the same buffer with a higher salt concentration. The salt concentration of the eluted samples is reduced by dialysis or ultrafiltration.

Another example of selective adsorption is the one proposed [136] for human urinary kallikrein preconcentration by foam fractionation. This is a type of adsorptive bubble separation technique (adsubble technique [137]) in which surface-active substances, such as proteins and enzymes because of their strong amphipathic nature, are removed from the solution by selective adsorption at the surface of bubbles rising through the solution forming a foam at the top of the solution. In spite of work done on foam fractionation of proteins and enzymes [138], demonstrating the usefulness of the technique, the latter has only been used for human urinary kallikrein preconcentration.

Lastly, some adsorbents have been proposed for kallikrein enrichment from human urine [139–141]; among these, chitosan and arginin-Sepharose seem to give the highest yields: 80% and 60%, respectively [136].

3.2. Purification of kallikreins

3.2.1. The concentrated extract

The complete composition of the extracts is not known, as the specific separation problem is considered each time, taking into account the form of the kallikrein sought. Variable amounts of proteins, some of which are serine proteinases, occur in the concentrated extract, even though they are not necessarily all those present in the original material because of the removal of a part of them (e.g. Tamm-Horsfall protein from urine) during the sample preparation stage [31,32].

In plasma, for example, HF, HK, Factor XI, α_2-glycoprotein I and immunoglobuln G (IgG) are

contaminants of prekallikrein purification; in particular, even though the addition of polybrene assures the inhibition of HF, the removal of the latter and of HK as soon as possible is in any case convenient to minimize the possibility of zymogen activation. For this purpose, the naturally occurring kallikrein inhibitors, HF and HK have been adsorbed on an anion exchanger [27,28]. The other contaminants are then removed step by step, in accordance with the purification technique adopted.

Owing to their dissimilar composition, different procedures have been proposed for the various types of extracts, even though homologous proteins of the kallikrein system are sufficiently alike to allow the use of at least some of the same methods in their purification. However, it is this great likeness among proteinases of the kallikrein family that seriously hampers their separation and characterization when they coexist in the same tissue (e.g. rat SMG or prostate). They are difficult to differentiate with polyclonal antisera and their molecular masses are also very similar, but the net electrical charge among them can be rather different with the result that anion-exchange chromatography has been proposed as the initial step (rat SMG) [142] and isoelectrofocusing for fractionation (rat prostate) [54].

Lastly, it should be noted that from the same concentrated extract other proteins besides kallikreins can be usefully obtained (e.g. ribonuclease A, trypsin, elastase and chymotrypsin from porcine pancreas). Various authors [132,143–145] have turned their attention to this problem, even recently.

3.2.2. *Strategies of purification steps*

Bearing in mind that the capacity and selectivity of the methods depend on the amount of contaminants, the volume to be dealt with and the purity required, two types of techniques which meet kallikrein purification requirements stand out: chromatographic and electrophoretic.

In view of limitations due to the stability of kallikreins under mild conditions of pH, a limited range of chromatographic principles have been applied in their purification schemes. In order to simplify the discussion, the analytical conditions of chromatographic and electrophoretic purification steps of certain interesting procedures are summarized in Table 3.

Operations such as ultrafiltration, dialysis, and freeze-drying, which in the original papers appear interposed between two purification steps, to make the sample composition suitable for the next one, have been omitted. After each step of the purification, assays for the kallikrein of interest and its purity are required to identify the fractions containing the enzyme prior to pooling for the next step and to assess the efficiency of the purification. In the various steps, protein concentrations were evaluated by absorbance at 280 nm or by well-known spectrophotometric procedures, bovine serum albumin being used as a standard [146–148].

Chromatographic methods. The chromatographic steps in Table 3 consist of low-pressure chromatographic systems and also of high-performance liquid chromatography (HPLC) apparatuses.

Low-pressure chromatography. As regards the principles applied in low-pressure chromatography, although there are exceptions, the preferred sequence consists of ion-exchange, followed by affinity chromatography with a final gel permeation step.

Ion-exchange chromatography. Ion-exchange chromatography can be useful as the first step to remove many contaminants which are adsorbed to the column, whereas the kallikrein form of interest does not adhere to the resin and is found in the unadsorbed effluent fraction. Furthermore, it is known that in order to bind a species to an ion-exchanger a low ionic strength is necessary. According to these criteria, in procedure (1) maximal adsorption of acidic proteins (including HF) to DEAE-cellulose was achieved by dialysing, against a buffer of low ionic strength, the filtrate deriving from a batch adsorption of human plasma on DEAE-Sephadex A-50. The exclusion in this step of acidic proteins was essential for an effective adsorption of prekallikrein to the affinity column in the following step. A similar task is performed by the cationic-exchanger in the first step of procedure (6).

In other instances, ion-exchange has been successfully used for fractionating groups [50,72] of kallikreins or isolating individual enzymes [37,46]. As tissue kallikreins carry a negative charge at the almost neutral pH required to protect biological activity, anion exchangers are often used. Besides their use upstream, to capture kallikrein or unwanted

Table 3
Chromatographic and electrophoretic techniques in some kallikrein purification procedures

Procedure	Source and isolated enzyme(s)	Purification steps 1	2	3	4	5	Yield (%)	Ref.
1	Human plasma prekallikreins I and II	DEAE-Cellulose 0.01 *M* TB, 0.01% Polybrene, 0.1% NaN_3, pH 7.0, r.t.	Arg-TADod-agarose 0.01 *M* TB, pH 7.0 0.02 *M* HAc*	CM-Sephadex C-50 0.1 *M* AmAc, 0.25 *M* NaCl, pH 5.0 0.1–0.4 *M* AmAc, pH 5.0, in 0.25 *M* NaCl*	Sephadex G-150 0.1 *M* AmAc, 0.15 *M* NaCl, pH 5.0	Isoelectric focusing column pH 7–9.5; 8–9.5 sucrose gradient 0–2°C	8,6	[28]
2	Human urine kallikreins A, B, D	DEAE-Cellulose DE-52 0.01 *M* PB, 0.2 *M* KCl, pH 7.0 0.2–0.4 *M* KCl, 0.01 *M* PB, pH 7.0*	Hydroxylapatite 0.01 *M* PB, 3 *M* NaCl, pH 6.0 0.01–0.08 *M* PB, 3 *M* NaCl, pH 6.0*	Aprotinin-Sepharose 0.05 *M* PB, 1 *M* NaCl, pH 7.0 1 *M* BA-HCl, 0.05 *M* PB, 1 *M* NaCl, pH 7.0*	Sephacryl S-400 0.025 PB, 0.5 *M* NaCl, pH 7.0		13(A) 27(B) 3(D)	[34]
3	Human urine *latent kallikrein*	Aprotinin-CH-Sepharose 0.05 *M* TB, 0.5 *M* NaCl, pH 8.0	QAE-Sephadex A-50 5 m*M* TB, 0.1 *M* NaCl, pH 8.0 0.1–1 *M* NaCl, 5 m*M* TB pH 8.0*	Hydroxylapatite 1 m*M* PB, pH 7.0 1–50 m*M* PB, pH 7.0*	TSK IEC DEAE 3SW 0.01 *M* PB, pH 7.0 KCl gradient to 1.0 *M* 0.01 *M* PB, pH 7.0*	Sephadex G-100 0.05 *M* TB, 0.2 *M* NaCl, pH 8.0	6,5	[32]
4	Bovine pancreas kallikrein B	DEAE-Sephadex A-50 0.05 *M* TB, 0.1 *M* NaCl, pH 7.4 0.05 *M* TB, 0.1–0.7 *M* NaCl, pH 7.4*	BA-Sepharose 6B 0.05 *M* TB, 25 m*M* NaCl, pH 7.4 0.025–0.7 *M* NaCl, 0.05 *M* TB, pH 7.4*	Sephadex G-150 0.05 *M* TB, 0.5 *M* NaCl, pH 7.4	Phenyl-Sepharose CL-4B 0.05 *M* TB, 4 *M* NaCl, pH 7.0 0.05 *M* TB, pH 7.0*		58	[72]
5	Guinea pig plasma prekallikrein	DEAE-Sephadex A50 20 m*M* TB, 40 m*M* NaCl, 3 m*M* EDTA 5% Polybrene, 3 m*M* BA, pH 8.0	CM-Sephadex C50 50 m*M* AcB, 0.1 *M* NaCl, pH 6.0	Isoelectric focusing column pH 3.5–11 sucrose gradient	Sephadex G150 20 m*M* TB, 1 *M* NaCl, pH 7.0		18.1	[68]
6	Porcine pancreas α-kallikrein B′	SP-Sephadex C-50 0.1 *M* AmAc, pH 5.4	DEAE-Sephacel 0.1 *M* AmAc, pH 6.7 0.1–0.6 *M* AmAc*	Hydroxylapatite 0.01 *M* PB, 1 m*M* $CaCl_2$, pH 7.0 0.01–0.07 *M* PB, pH 7.0 1 m*M* $CaCl_2$*	DEAE-Sepharose 0.05 *M* PB, pH 7.0 2 *M* KCl, 0.05 *M* PB, pH 7.0*	Sephadex G75 0.1 *M* AmAc, pH 6.7 follow two chromatographic steps on DEAE-Sephacel, *equilibrated and eluted as in* in step 2	12	[114]
7	Rat urine kallikreine	DEAE-Sepharose CL-6B 0.05 *M* TB, pH 8.0 0–0.5 *M* NaCl, 0.05 *M* TB, pH 8.0*	Sephadex G100 0.05 *M* TB, pH 8.0	BA-Sepharose CL-6B 0.05 *M* TB, 0.5 *M* NaCl, pH 8.0 0.1 *M* AcB, 1 *M* NaCl, pH 4.8*			60	[66]
8	Rat SMG rK2, rK9	DEAE-Sephadex A50 0.01 *M* PB, 0.15 *M* NaCl, pH 6.35 u.t.	Mono S HR 5/5 25 m*M* PB, pH 6.0 0–0.3 *M* NaCl, 25 m*M* PB, pH 6.0, u.t.*					[53]
9	Rat prostrate rK9 isoenzymes	DE-52 Whatman 0.05 *M* AmAc, pH 5.8	SBTI-Sepharose 4B 0.02 *M* PB, 0.5 *M* NaCl, pH 7.0 0.1 *M* AcB, pH 3.5*	Mono P HR5/20 0.075 *M* TB, pH 9.3 *Polybuffer 96 (pH 6.0)* 22°C*	Mono S HR5/5 0.05 *M* AmAc, pH 4.0 0.5 *M* AmAc, pH 4.0, 22°C*			[52]
10	*A.h. ussuriensis* venom kallikrein-like	Accell Plus QMA 0.05 *M* TB, pH 8.0 0–0.25 *M* NaCl, 0.05 *M* TB, pH 8.0, u.t.*	Protein-Pack DEAE 15HR 0.05 *M* TB, pH 8.0 0–0.25 *M* NaCl, 0.05 *M* TB, pH 8.0, u.t.*				13,5	[78]
11	*L. muta* venom kininogenin	Sephacryl S-200 0.05 *M* NaCl, r.t.	Sephadex G-75 distilled water, r.t.	Flatbed isoelectric focusing pH 2.6–9.8	Preparative PAGE 7–13% gradient polyacrylamide gel slab		2,1	[79]

All steps were carried out at 4°C unless otherwise indicated.

* Elution conditions.

Abbreviations: Arg-TADod = L-arginyl-triazinyl-aminododecyl; TB = Tris–HCl buffer; AmAc = ammonium acetate; AcB = acetate buffer; r.t. = room temperature; HAc = acetic acid; PB = phosphate buffer; u.t. = unspecified temperature.

components, ion exchangers appear in various schemes also downstream, as a polishing step [69,114,124,126]. In procedure (9), outlining the separation of very similar molecules such as isoenzymes, the simple, rapid technique of chromatofocusing has been successfully applied.

Affinity chromatography. Another critical feature exploited to fractionate and purify kallikreins is their different sensitivity to various inhibitors [149,150]. A number of biospecific ligands have been proposed to adsorb selectively and reversibly the various kallikrein forms; Table 4 presents a survey of affinity chromatographic techniques applied to kallikreins. In some instances, an improvement in the purification has been achieved [152,153] by the interposition of a spacer arm to distance the ligand from the matrix backbone, thus overcoming steric limitations. Aprotinin is one of the inhibitors [166] most frequently used in affinity chromatography for kallikrein purification; for this, as for other ligands [SBTI, benzamidine (BA)], the elution of the enzyme adsorbed, after eliminating the contaminants by washing, is frequently accomplished by pH lowering. Considering the above reported instability of kallikreins at low pH values, in the steps (Table 3) involving elution from affinity columns, the eluate fractions were collected and brought to a safe pH value (5–7). Affinity chromatography with more common ligands generally follows ion-exchange, i.e. when many contaminants have been removed; it rarely appears early in a purification scheme. In procedure (3) the need to separate the latent form of the enzyme from active kallikrein and other unwanted components has suggested the affinity step upstream (repeated twice). Immobilized antibodies [58,64] and overall monoclonal antibodies [63,134] are among the most selective and powerful ligands used for the purification of kallikreins, either bonding these enzymes or the contaminants. The particular resistance of antibodies to various kinds of attack from different components of the samples enables them to be used early in a purification procedure.

Hydrophobic interaction chromatography. As is known, the Achille's heel of affinity chromatography is the difficulty of a large-scale application of the technique due to problems in predicting column behaviour after scaling-out and the lack of the right solid matrix supports for fast flow operations. Apart from this, it has been reported [167] that in rat SMG kallikrein purification, a simple procedure involving hydrophobic interaction chromatography was superior to both aprotinin-affinity chromatography and immunoaffinity chromatography. The procedure utilising the hydrophobic resin compared with aprotinin-affinity resin, was of lower cost and did not subject the kallikrein to the potentially denaturing conditions of a low pH; in comparison with immunoaffinity a higher recovery was achieved.

Although not very recurrent among the chromatographic principles applied to kallikrein purification, hydrophobic interaction chromatography provides an interesting additional means of separation with the valuable characteristic of not requiring a previous adjustment of the ionic strength.

Adsorption chromatography on mineral polymers. As well as hydrophobic interaction, hydroxyapatite chromatography can be used at a pH value close to the physiological one and, therefore, this technique is suitable for kallikrein fractionation. Some examples of applications appear in Table 3. It is worth mentioning that in the purification of kallikrein of human saliva this purification has been omitted [96], compared in the earlier adopted procedure [168,169], due to the possible loss of the enzyme activity.

Gel permeation chromatography. Besides its use to determine the relative molecular mass of a number of kallikreins (Table 1), gel permeation has been helpful in kallikrein concentration in preparative procedures, however, the resolution may not be convenient for upstream use when kallikrein complex matrices are handled and in addition, this technique is difficult to use when dealing with large volumes; consequently, gel permeation is used rather as a terminal polishing step.

Furthermore, in some instance (rat pancreas) it can be essential to remove most of the A_{260} absorbing material (nucleotidic contaminants); this can be performed by gel permeation.

High-performance liquid chromatography. The speed and efficiency of HPLC processes has led to an advantageous application of these techniques in kallikrein purification (Table 3, procedures 8, 9, 10). In particular, only two HPLC systems in series allowed (procedure 10) the purification of a kallikrein-like enzyme in a few hours, compared with several days required by conventional chromato-

Table 4
Further affinity chromatography techniques applied to kallikrein purification

Source	Ligand	Eluent	Ref.
Human plasma	anti-(human IgG)*	–	[151]
	HK	3 *M* NaSCN	[29]
	protein A*	–	[81]
	anti-kallikrein IgG	0.1 *M* GlyB, pH 2.5	[30]
Human serum	SBTI with	0.1 *M* PB, 0.2 *M* NaCl,	[153]
	polysaccharide spacers	pH 6.2	
Human urine	Aprotinin	0.1 *M* AcB, pH 3.5	[154]
	PABA	0–0.35 *M* Gn-HCl	[31]
	Affi-Gel Blue*	–	[31]
	anti-kallikrein IgG	pH gradient	[155]
	anti-prokall. IgG*	–	[155]
	anti-kallikrein IgG	4 *M* NaSCN	[156]
	Aprotinin	0.2 *M* KCl, HCl, pH 3	[156]
Human pancreas	Aprotinin	0.1 *M* AcB, pH 4.0	[35]
Human SMG	PABA	0–0.3 *M* Gn-HCl	[157]
	BA	1.0 *M* Gn-HCl	[158]
Porcine urine	Aprotinin	0.3 *M* BA	[114]
Porcine SMG	guanidinated Aprotinin	0.5 *M* BA	[114]
Porcine pancreas	triazin-benz. deriv.	0.5 *M* KCl	[159]
	bis-cat. triazin-benz. deriv.	0.5 *M* KCl	[160]
	Leupeptin	0.2 *M* Leupeptin	[161]
	PABA	0.1 *M* $NaHCO_3$, 1 *M* NaCl	[132]
Rat plasma	SBTI	5 m*M* NaOH	[47]
	Peptidyl-SC deriv.	1 *M* Gn-HCl,1 *M* NaCl	[162]
	Aprotinin	50 m*M* PABA, 0.1 *M* TB, pH 8	[163]
Rat stomach	PABA	1.0 *M* NaCl	[48]
Rat skeletal muscle	monoclonal antibody	0.1 *M* acetic acid	[134]
Rat heart	Aprotinin	0.1 *M* Gly/HCl, pH 3.0	[49]
Rat SMG	anti-kallikrein K1*	–	[58]
	Aprotinin	0.05 *M* formic acid	[51]
	Aprotinin	0.1 *M* AcB, pH 3.5	[59]
	Aprotinin	1 *M* BA	[130]
Rat pancreas	anti-prokallikrein	0.5 *M* NaCl, AcB, pH 4.0	[61]
Rat prostate	Aprotinin	HCl, pH 2	[164]
Rat spleen	monoclonal antibody	0.1 *M* acetic acid	[63]
Rat adrenal glands	anti-kallikrein	1.0 *M* NaCl, AcB, pH 3.5	[64]
Rat urine	Aprotinin	HCl, pH 2	[165]
Dog pancreass	Concanavalin A	0.15 *M* a-methyl-mannos.	[74]
	Aprotinin	0.1 AcB, pH 4.0	[73]
Rabbit urine	Aprotinin	1.0 *M* BA	[75]
Vipera a. aspis venom	Heparin	0.3 *M* NaCl	[80]

* Ligand of unwanted components.
Abbreviations: GlyB = glycine buffer; PB = phosphate buffer; AcB = acetate buffer; Gn = guanidine; benz. = benzamidine; deriv. = derivatives; cat. = cationic; SC = semicarbazone.

graphic steps. Examples of HPLC techniques applied to kallikrein purification are reported in Table 5.

Electrophoretic procedures

In procedures (1) and (5) of Table 3 the final steps include isoelectric-focusing, a very attractive technique especially for isolation purposes, because of its excellent resolution and load capacity. It is particularly useful when most of the contaminants have been removed by the previous steps. Without entering into the aspects of the technique, which have been exhaustively discussed by various workers

Table 5
Examples of HPLC techniques applied to the purification of kallikreins

Technique and kallikreine source	Column	Ref.
High-performance size exclusion chromatography		
Mouse SMG, tissue kallikrein	TSK-G3C1OOSW (Tosoh)	[46]
Rat SMG	Superose 12 (Pharmacia)	[57]
High-performance ion-exchange chromatography		
Human urine, latent kallikrein	TSK IEC DEAE 3SW (Kratos)	[32]
Human salivary-gland prokall.	Fractogel EMD-TMAE650(S) (Merck)	[37]
Mouse SMG, tissue kallikrein	Mono Q (Pharmacia)	[46]
Rat SMG: rK1, rK2, rK7, rK9, rK10	Mono P (Pharmacia)	[59,145]
	Mono S (Pharmacia)	[53,56]
	Mono Q (Pharmacia)	[55,145]
Rat prostate, rK9 isoenzymes,	Mono P (Pharmacia)	[52]
rK8	Mono S (Pharmacia)	[52]
A.h. ussuriensis venom, kall.-	Accell Plus QMA (Nlillipore)	[78]
like	Protein-Pack DEAE 15HR (Millipore)	[78]
C. basiliscus basiliscus venom,	Aquapore CX-300 (Brownlee)	[170]
Reversed-phase high-performance chromatography		
Mouse SMG, tissue kallikrein	ODS-120T (Tosoh)	[45]
Rat SMG	BU 300	[51]
High-performance hydrophobic interaction chromatography		
Human urinary prokallikrein	TSK Phenyl-5PW (LKB)	[31]

[171–174], it is convenient to recall that at the end of the focusing step, kallikrein has to be recovered by rapid, simple, non-destructive methods. Thus, elution from gel, gel permeation, affinity chromatography and concentration by freeze-drying of the isolated fractions are all steps which can be involved. Polyacrylamide gel electrophoresis (PAGE) is a method that has been used for a long time, which makes it possible to separate intact kallikrein for a later check of biological activity. The simplest forms of PAGE based on uniform polyacrylamide concentration and a single homogeneous buffer system (continuous zone electrophoresis), have been largely replaced by the techniques known as 'disc electrophoresis' (with reference to the discontinuous buffer systems used). These last techniques, successful for their inherently high capacity to obtain very sharp protein bands, have been used in procedures of prekallikrein or kallikrein characterization.

4. Detection methods

Kallikreins can be rapidly assayed spectrophotometrically by esterolytic and/or amidolytic assays, though problems may arise because of other interfering components [175].

If an appropriate antibody is available, immunological assays are also used [82,84,176–181]: they are not usually as quick as enzyme activity assays and do not distinguish between active and denatured forms of the enzyme. However, these types of tests may be highly sensible and many samples can be quite easily screened [156].

The strictly biological determinations (e.g., the reversible reduction of blood pressure in animals) are relatively sensible and highly specific, but very time-consuming, cumbersome, and inconvenient for routine purposes [156].

A comparison of amidolytic, coagulation and immunochemical assays of prekallikrein in human plasma has been reported [182].

Purity is often assessed by SDS electrophoresis, a single band being usually accepted as an indication of a pure enzyme. For the various forms of kallikreins, which do not give a single-band, the criterion of purity is when none of the bands disappears as a result of further purification steps.

HPLC has been used to provide confirmation of homogeneity, by a sharp single peak, of purified kallikrein or kallikrein-like enzyme [183–187].

4.1. Methods applicable to HPLC and gas chromatography (GC)

4.1.1. Detection by enzymatic activity

The kinins released from purified kininogens as substrates can be determined by chromatography. It is worth mentioning that kallikreins release kallidin/bradykinin, two kinins very different in basicity and hydrophobicity, from kininogen. The early analytical procedures for the separation of these kinins, based on ion-exchange chromatography, took a long time. Now, this separation is quite easy by HPLC and some methods for the determination of kallikreins through kinins released have been described [188–190].

In synthetic substrate assays, the esterolytic or amidolytic properties of kallikreins are exploited [191–197]. These assays are easy to perform and have a relatively high sensitivity, but most of the substrates used may also be cleaved by other trypsin-like enzymes. Therefore, kallikrein activity measured with synthetic substrate is often confirmed by inhibition of enzymatic activity with specific inhibitors.

Both HPLC and GC have been used to determine the products resulting from the esterase activity of kallikrein on the substrate selected.

The HPLC method [198] is based on the separation of benzoylarginine ethyl ester (BAEE) and benzoyl-L-arginine on a reversed-phase column, followed by their determination using an external standard. The elution patterns resulting from injections in the HPLC apparatus and isocratic elution have shown that in time-course experiments, the rate of hydrolysis is linear within 10 min, and that the initial rate of hydrolysis of the substrate catalyzed by the enzyme is proportional in the range 2–7 μg of kallikrein.

The detection limit of the latter has been reported as 1 μg/ml. The method, assayed on commercial samples, has given results which agree with those obtained by the titrimetric method (differences of about 1.8%).

The GC method [199] determines the ethanol resulting from the esterolytic activity of human urinary kallikrein using Ac-Phe-Arg-OEt as the substrate. The samples were centrifuged and then dialyzed to avoid any contamination with alcohol, and to eliminate the interfering volatile substances present in the matrix. After incubation in a vial, the alcohol was determined by head-space chromatography. The method shows a high mean recovery (97%) and precision; the detection limit is 0.5 ng of kallikrein, but it could be lowered by using enrichment precolumns based on cold trapping techniques.

More reliable measurements of kallikreins are achieved by the chromatographic signals from the eluted kallikrein forms; various methods for the quantitation of eluted kallikreins corresponding to direct and specific chromatographic signals have been described.

A rapid separation and measurement of rat urinary kallikrein HPLC using an ion-exchange column has been proposed [200]. The urine after centrifugation and lyophilization is directly chromatographed and the peptidase or the esterase activity is continuously monitored using a post-reactor system and appropriate substrates. The detection of the enzyme by peptidase activity is superior to that by esterase activity and a linear relation between peak area and sample volume has been found.

4.1.2. HPLC simultaneous determination of kallikrein and other proteinases

In recent papers [201,202] the possibility has been examined of kallikrein determination in the presence of other proteinases. The proposed method allows the simultaneous determination of molar amounts of active proteinase molecules. This approach to the quantitative determination of serine proteinases avoids the use of the substrate, takes advantage of the chemical stability and ready availability of aprotinin as a reagent and does not involve laborious chemical modifications of the inhibitor. The limit of determination was found to be 0.1 nmol for the enzymes in the original sample. The method depends on the formation of stable complexes between aprotinin and enzymes, and on their chromatographic discrimination.

The previous method for determining more than one serine proteinase simultaneously by hydrophobic interaction chromatography of their complexes with aprotinin is inapplicable when other UV-absorbing species are co-eluted. Therefore a new procedure has been described which, without introducing changes in the chromatographic separation, allows direct determination of serine proteinases in terms of molarity. The suitability of ^{125}I-labelled aprotinin has therefore been tested as a reagent in the analysis of

mixtures containing various enzymes taken as models (Fig. 1, Fig. 2a,b). The exclusion of interferences, which trouble the UV chromatographic profile, allows an easy, fast simultaneous determination of three serine proteinases. The radioactivity measured for each complex is directly related to the amount (nmol) of aprotinin and hence to an identical amount of the enzyme, owing to the 1:1 stoichiometry of the complex.

4.1.3. Detection by immunological assays

A determination [85] of both active and inactive rat urinary kallikreins has been developed by using specific radioimmunoassay (RIA) after HPLC separation, with a recovery of 79±11%. Rat urinary kallikrein, purified and characterized by HPLC analysis, PAGE electrophoresis and electrofocusing has been used both to raise antibodies in rabbits and to be labelled with ^{125}I. In order to measure the concentration of both kallikrein forms, these are collected by anion-exchange HPLC separation. The incubation of labelled antigen is performed in an appropriate medium, with standard or unknown samples, in the presence of dilute antiserum; after separation from [^{125}I]kallikrein and centrifugation, the radioactivity of the unwashed precipitate is counted with a γ-spectrometer. The detection range is from 0.125 to 16 ng of kallikrein.

4.2. Methods applicable to electrophoresis

SDS-PAGE plays a very important role in the determination of the relative molecular masses of kallikreins and consequently its use is mentioned in

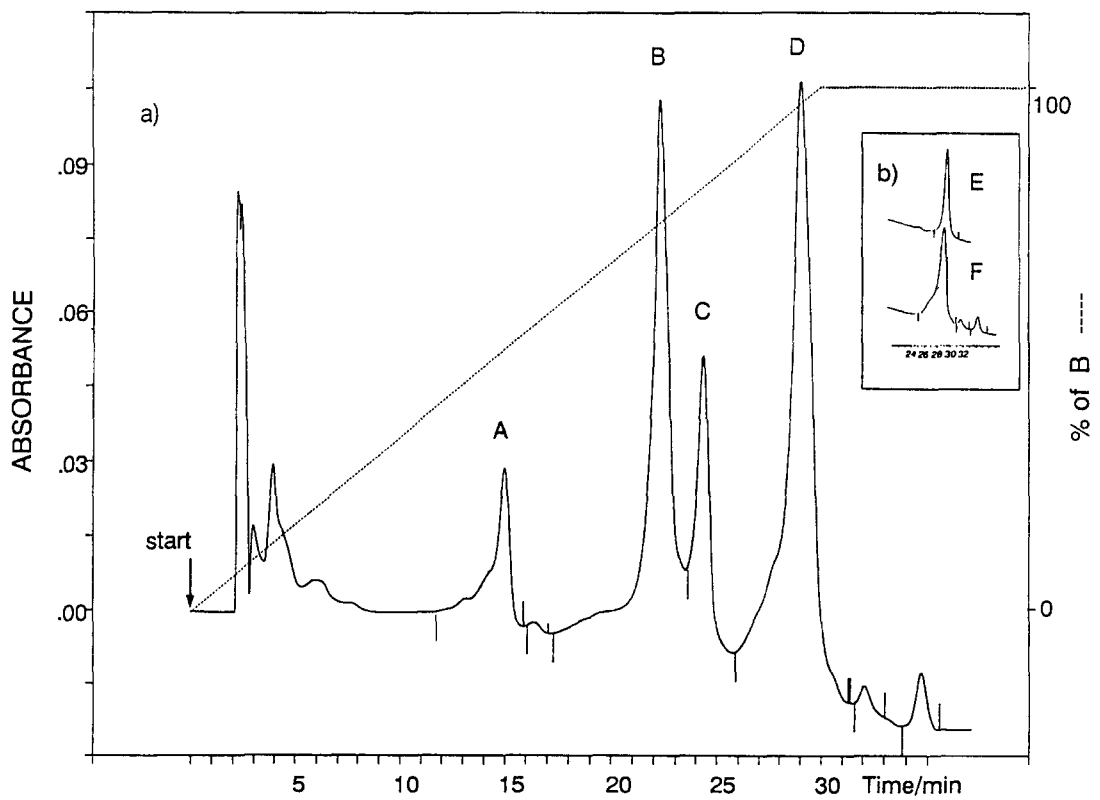

Fig. 1. (a) Chromatogram of enzyme mixture containing ribonuclease, 5.0 nmol (A), lysozyme, 5.0 nmol (B), trypsin, 5.0 nmol (C) α-chymotrypsin, 5.0 nmol and kallikrein, 1.0 nmol (D), brought to 1.0 ml with buffer solution A. (b) Separate chromatograms of α-chymotrypsin, 2.5 nmol (E), kallikrein, 3.2 nmol (F). Chromatographic conditions: Bio-gel TSK Phenyl 5PW column (75×7.5 mm); flow-rate, 1.0 ml/min with 30-min linear gradient ammonium sulphate 1.8 to 0.0 *M* in 0.1 *M* sodium phosphate buffer (pH 7.0); volume injected 100 μl; λ=220 nm; % B indicates % buffer B (0.1 *M* sodium phosphate buffer pH 7.0) (from [202], with permission).

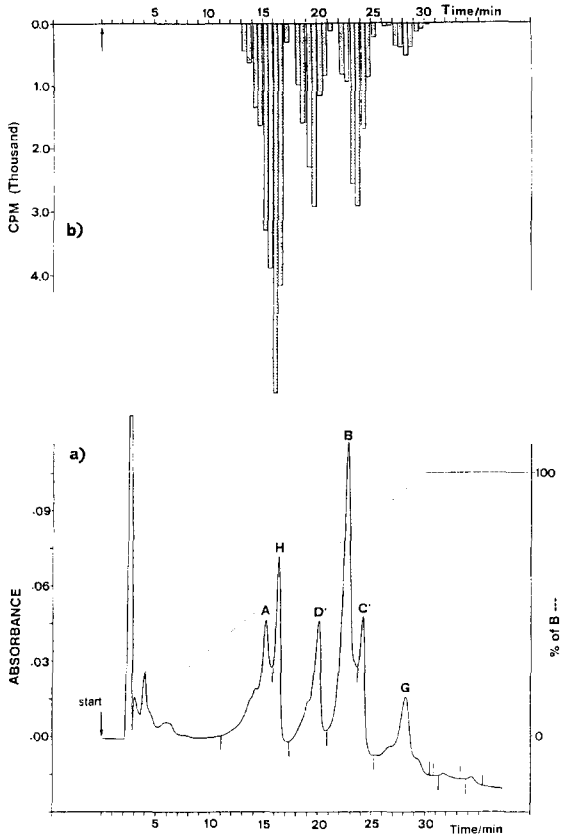

Fig. 2. UV absorbance and radioactivity registrations of same enzyme mixture as Fig. 1 after addition of (a) aprotinin, 22.0 nmol, ribonuclease (A), residual aprotinin (H), lysozyme (B), aprotinin–trypsin complex (C'), aprotinin–α-chymotrypsin complex (D') and aprotinin–kallikrein complex (G). (b) ^{125}I-labelled aprotinin 22.0 nmol, (2000 cpm/nmol). Chromatographic conditions as Fig. 1. (From [202], with permission.)

most purification reports (Table 1). Generally performed in one dimension using the discontinuous Tris–glycine buffer system, it is sometimes carried out in two dimensions [74] for better separation and detection sensitivity by lowering the background noise. The addition (1–5%) of reducing agents (e.g. dithiothreitol or 2-mercaptoethanol) in several instances allow an explanation of the role played by disulphide bonds in retaining one of two (high and low) polypeptide chains (Table 1).

In isoelectric focusing the p*I* of the purified enzyme is frequently estimated by using commercial polyacrylamide gels (nominal pH range 3–10) at 4°C, 200 V with a possible drop to 1500 V [124]. The technique is usually employed to characterize the final purified kallikrein product rather than for monitoring the progress of purification steps. Isoelectric focusing and SDS-PAGE, when used in a parallel analysis of a sample, form a powerful method to assess kallikrein charge and size heterogeneity, respectively [28,52].

4.2.1. Staining procedures

For the analysis of electrophoretic profiles, after gel fixation, the staining method generally involves an irreversible alteration of the kallikrein enzyme to observe the single or various bands formed [203,204]. Thus, Coomassie Brilliant Blue R250 and sometimes silver staining are used: the latter, 20–200 times more sensitive than the former, can be preferred for the detection of trace impurities in the purification steps, provided that various chemicals (e.g. acetic acid, agarose, Tris) frequently used in electrophoretic procedures are removed from the gel before staining. Taking into account that kallikreins are glycoproteins, the periodic acid–Schiff's base method is sometimes employed [205,206].

A novel way [207] to determine relative molecular masses of proteinases, including tissue and plasma kallikreins, uses a relatively simple SDS-PAGE–autoradiography procedure. The gels contain copolymerized radiolabelled substrates which, after electrophoresis, are cleaved in situ from the reactivated enzyme. The resulting smaller peptides are able to diffuse out of the gel, leaving clear areas against a dark background by autoradiography.

The detection of kallikreins on the basis of their enzymic activity has also been recently exploited by using the enzyme overlay membrane technique [208]. Proteins from homogenate (rat SMG) were focused (pH 4–6.5) and the proteinases were visualized under long-wave UV light, by overlaying the focused gels with cellulose diacetate membrane impregnated with solutions of the fluorogenic oligopeptide proteinase substrates D-Val-Leu-Arg-AFC and Z-Val-Lys-Lys-Arg-AFC.

4.2.2. Immunodetection

The identification and characterization of kallikreins separated by electrophoretic procedures has taken considerable advantage of the specificity and

affinity of antibodies. These substances can directly precipitate the enzyme in the gel matrix (immunofixation), but it is usually preferable to use Western, Southern or Northern blotting, which are the most modern and widely-used techniques in the biological field [209–214].

Blotting techniques

When the bands have been transferred on to the membrane, different kinds of determinations can be accomplished, including autoradiography or immunoreaction with specific antibodies [215–218].

By a combination of Western blotting, SDS-PAGE and HPLC techniques, two intermediates have been identified in the formation of bradykinin from HK by plasma kallikrein [219]. By using standard immunoblot techniques, it has been shown [220–222] that a monoclonal antibody (MAb 13G11) specifically recognizes the two variants of human plasma prekallikrein separated by SDS-PAGE and also the complexes formed by kallikrein and the inhibitors (Cl-Inh and α_2M) present in plasma. Though the immunoblot assays have the potential of quantitating prekallikrein, kallikrein and kallikrein–inhibitor complexes in plasma, they appear to be more useful for investigating the general pattern of distribution of the products resulting from prekallikrein activation, as well as for detection of abnormal compounds that form SDS-resistant complexes with kallikrein.

Immuno-probing of nitrocellulose blots of SDS-PAGE gels run with rat SMG extracts and isoelectric focusing have been used [223] in the study of the influence exerted by the animal sex and type of autonomic nerve impulse on the secretion of multiple forms of tissue kallikrein. Northern blot analysis has revealed that a kallikrein-binding protein mRNA is located mainly in the medulla of rats; Northern and Southern blots have allowed the study of kallikrein gene expression in human tissues and in a rat model of hypertension; Southern blot analyses have been performed in the identification and structure of the rat true tissue kallikrein gene.

'Amidoblot' is the name given to a technique [224] by which amidolytically active prothrombin activation products are sensitively detected after SDS-PAGE, blotting onto a nitrocellulose membrane, and visualized with a chromogenic substrate. Human plasma kallikrein can be visualized by S2302 (D-Pro-Phe-Arg-pNa) as a substrate.

4.3. Direct assays without separation steps

The interest for direct assays of kallikrein in tissues extracts and body fluids, in view of physiological and pathological studies, is evident. It is worth mentioning that kallikrein is present in such complex materials in different forms, such as catalytically active enzyme, enzymatically inactive proenzyme, complexes with inhibitors. Due to the fact that no single assay technique can accomplish the quantification of each of above forms, specific and sensible analytical methods are, of course, required. The multistep procedures utilized for kallikreins with preparative or characterization purposes (involving unavoidable, irreproducible losses) cannot be employed and sample clean-up is reduced to a minimum.

Several different methods have been developed which include procedures using synthetic substrates, kinin-liberating assays and immunoassays; some of them are reported in Table 5. Furthermore, procedures concerning the localization of kallikrein in various tissues by immunohistochemical methods have been reported [225].

4.3.1. Synthetic and biological substrate assays

Inactive forms cannot be directly detected by these methods. An estimate of the proportion of proenzyme in a sample is obtained by performing the assays before and after suitable activation. As already stated, all enzymic methods are subjected to interference by inhibitors and trypsin-like enzymes present in the tissue extracts and body fluids.

Single peculiarities characterize the various procedures. For example, the advantage of the Ac-Phe-Arg-OEt assay [226] is the possibility of continuous monitoring during the test, while a drawback is the alcohol sensitivity of the reaction. This is not found in the D-Val-Leu-Arg-pNA assay [227], but this method suffers from the disadvantages of a lower sensitivity.

In several instances the activity of kallikrein can be hardly detected by hydrolysis of synthetic substrates, because of the low concentration of enzyme. In such cases methods such as enzyme- and/or

radioimmunoassay are used. In this context applications studies of other synthetic substrates appear in the literature [191–193].

In biological substrate assays, the kinins released from purified kininogens as substrates can be determined by radioimmunoassay, enzyme immunoassay or HPLC (see Section 4.1.1). The methods are very sensible, but the preparations of kininogen with high activity and of antibody against bradykinin are laborious. Moreover [228], it is often difficult to obtain specific antibody with high titer and kinin antibody invariably shows recognition for kininogen.

However, the activity of about 119 fg (2.6 attomol) of kallikrein in rat urine can be detected by a microkininogenase assay [229]. The technique involves the removal of unreacted bovine or dog kininogen and kinin-RIA analysis. Recently an assay has been described [181] to detect kallikrein from superfused isolated rat renal cortical cell. By using human kininogen as substrate and RIA for quantitation, the assay sensitivity is 0.01 U/ml, equivalent to 3 ng/ml/min of generated kinin.

4.3.2. Immunoassays

Increasingly, immunoassays (generally radioimmunoassays) are being used to detect low concentrations of kallikrein. However, problems of different kinds can arise: polyclonal antibodies prepared against kallikrein may not distinguish between active and inactive forms of the enzyme, or between native and denatured enzyme. Technical problems are due to the need of purified kallikrein for immunization: if the enzyme is not available, or its storage is difficult, it is inevitable that purification procedures have to be repeated over and over again. The techniques of obtaining monoclonal antibodies may require considerable time.

A method [135], developed for the immunoassay of glandular kallikrein, allows us to overcome the problems arising from the use of [^{125}I]-kallikrein as a

Table 6
Some direct assays for kallikreins

Method	Enzyme source	Detection limit (ng)	Ref.
Synthetic substrate assays			
Ac-Phe-Arg-OEt	Human urine	10	[226]
D-Val-Leu-Arg-*p*-NA	Human urine	50	[226,227]
	Human biological fluids	1[a]	[83]
D-Pro-Phe-Arg-[^{3}H]benzyl-amide	Human urine	5	[231]
Biological substrate assays			
Radio immunoassay			
Dog kininogen	Human biological fluids	0.02[a]	[83]
LK	Rat urine	0.001	[65]
Enzyme immunoassay	Human urine	3[b]	[232]
Immunoassays			
Radioimmunoassay	Porcine pancreas	0.04	[114]
	Porcine pancreas	0.3	[114]
	Rat urine	0.04	[65]
	Rat SMG	0.16	[61]
	Rat plasma	0.02	[233]
	Human serum	0.016	[234]
Enzyme immunoassay	Human urine	0.05	[156]
	Human biological fluids	0.04[c]	[83]
Bioluminescence immunoassay	Human urine	2.5×10^{-6}	[156]

[a] As enzymatically active kallikrein.
[b] As liberated bradykinin.
[c] As kallikrein/α_1-proteinase inhibitor complex, or as total kallikrein.

labelled tracer and to retain the excellent limits of RIA detection. The procedure, called labelled inhibitor-enzyme immunoassay, is based on the use of ^{125}I-labelled aprotinin as a tracer.

Lately, two immunoassays [83,230] have been proposed for the specific determination of different forms of human tissue kallikrein in body fluids. In the first (Table 6), a system of assays based on one monoclonal antibody allows the determination of total immunoreactive tissue kallikrein, of the complex of tissue kallikrein–α_1-proteinase inhibitor, and of enzymatically active tissue kallikrein. In the second, an enzyme-immunoassay was developed to determine tissue kallikrein (detection limit: 0.45 ng/ml) and α_1-antitrypsin–tissue kallikrein complexes (detection limit: 1.5 ng/ml).

5. Particular instances

Carbohydrate moieties of many different kallikreins constitute an area for research, because as yet no complete information has been reported on the nature and type of oligosaccharides present. On the other hand, from a general view-point the application to a therapeutic use of recombinant-derived glycoproteins has created a pressing need for mapping techniques for carbohydrate structures to confirm the consistency of glycosylation and to identify changes which may have occurred due to varying cell culture conditions or during manufacture [235]. Separation methods by HPLC [236–240], based on anion-exchange, size-exclusion, ion suppression, and reversed-phase chromatography have been widely used, because of the clear advantages offered by the technique. Recently, the human tissue kallikrein gene has been cloned and recombinant-derived glycoprotein expressed in Chinese hamster ovary cells in amounts sufficient for an evaluation of the type of glycosylation present [241]. The high pH anion-exchange chromatography, coupled with pulsed amperometric detection, has proven [242–244] a highly versatile and useful technique for determining carbohydrates.

A novel, sensitive method [245] to detect and compare the glycosylation of the individual kallikrein of rat SMG by means of lectin probes has been developed. After isoelectric focusing of the glandular extract and visualization of the bands by fluorogenic substrates, the proteinases were eluted and slot-blotted onto nitrocellulose membranes. The proteinase blots were probed with lectins having different sugar affinities: lectin staining demonstrated differentially glycosylated kallikrein isoforms.

To complete the excursus on different chromatographic applications involving kallikreins, we may remember: (a) immobilization of kallikrein (often as porcine pancreatic) on Sepharose to retain selectively aprotinin or other inhibitors in view of their purification or determination in biological fluids [246–248]; (b) kallikrein removal (with other trypsin-like enzymes which cause plasma protein instability) from plasma to facilitate the storage and transportation of plasma products [249–251]. For this purpose, plasma is passed through affinity cartridges, coupled with *p*-aminobenzamidine, by radial-flow chromatography. Radial chromatography is reported to have advantages in a low pressure drop at a fast flow-rate and in linear scaling-up. The removal of proteases from human plasma shows 70% efficiency after one-pass treatment.

6. Conclusion and perspectives

The determination of kallikreins even in ultramicroamounts in various biological matrices is of paramount interest for both physiological and clinical purposes because, as appears from literature, different forms of these enzymes seem to have a share in many biological processes which may lead to pathological conditions of different tissues and their exudates. For such determinations, which have to allow the evaluation of even very low values of concentration, the availability of separation procedures is essential for two reasons: (a) the immunological methods need selected standards; (b) the coexistence of very similar other forms of kallikreins.

In the last decade, a lot of work has been done in this direction with the participation of various HPLC and electrophoretic techniques; meanwhile the possibility has also grown of driving the limit of determination to lower and lower concentrations. The development of selective, sensitive methods can improve the future separation of the various forms of kallikreins to ameliorate the understanding of the biological phenomena attributable to them. Especial-

ly HPLC and capillary electrophoresis combined with MS detection are expected to be fruitful.

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