Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1079 (2005) 187–196
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Solid-phase capture and release of arginine peptides by selective tagging and boronate affinity chromatography

A. Foettinger, A. Leitner, W. Lindner *

Christian Doppler Laboratory for Molecular Recognition Materials, Institute of Analytical Chemistry, University of Vienna, Währinger Straße 38, 1090 Vienna, Austria

Available online 7 April 2005

Abstract

A method for the selection of arginine-containing peptides from a mixture by a solid phase capture and release technique is presented. The method is based on the covalent modification of the guanidine group of arginine with 2,3-butanedione and phenylboronic acid under alkaline conditions. Using polymeric materials with immobilised phenylboronic acid the arginine-peptides can be captured on a solid support while arginine-free peptides are not covalently bound and can be washed away. Finally, the arginine-peptides can be cleaved again from the boronic acid beads due to the reversibility of the reaction. The recovered peptides are then analysed by liquid chromatography–tandem mass spectrometry. The method was optimised with model peptides with regard to the non-specific binding of arginine-free peptides and quantitative cleavage of the label after the selection step. Using an adequate protocol, the applicability towards more complex samples was successfully tested with a tryptic digest of a mixture of three standard proteins.

Keywords: Arginine-peptides; Phenylboronic acid beads; Affinity enrichment; Tryptic digest; Mass spectrometry

1. Introduction

Proteomics is a rapidly growing field in the chemical and biological sciences. Gene expression does not always correlate with protein expression because many protein processing events cannot be predicted by genomic data alone. The identification of the proteins expressed in a cell can help to get an insight into biological processes and to explain biological phenomena.

Traditionally, protein mixtures have been separated by two-dimensional gel electrophoresis (2D-GE). The gel spots of interest are subjected to enzymatic digestion and the resulting peptides are then typically analysed by different mass spectrometric methods. For identification of the proteins the MS data (peptide masses and/or MS/MS data) is used for database search. However, GE-based methods have well known drawbacks. To overcome these problems liquid chromatographic methods either based on one-dimensional (reversed phase) or two-dimensional (e.g. strong cation exchange plus reversed phase) HPLC, combined with electrospray ionisation and tandem mass spectrometry gained popularity, especially for the analysis of more complex protein mixtures [1–3]. However, compared to 2D-GE separations, LC-based methods have a limited resolution when they are applied to this type of sample. In addition, the data acquisition speed of today's MS/MS platforms is still compromised, so that not all peptides eluting simultaneously from the column will actually be sequenced (fragmented), limiting the number of peptides that can be identified. Consequently there is still a need for methods to reduce the sample complexity while maintaining enough information for the identification of proteins. In this context chemo- and bio-affinity selection techniques have gained popularity and various methods have been developed over the last years [4–6]. They are directed to peptides containing certain amino acids or also post-translationally modified proteins, often in combination with stable isotope labelling to enable relative quantitation. Affinity targets can be either created by the addition of affinity groups on the analytes or

* Corresponding author. Tel.: +43 1 4277 52300; fax: +43 1 319 6312.
E-mail address: wolfgang.lindner@univie.ac.at (W. Lindner).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.03.038

by taking advantage of affinity targets already present in the analytes.

The probably most known method is the isotope coded affinity tag (ICAT) for the specific modification of cysteine residues [7]. The affinity tag contains a biotin moiety, allowing sample fractionation by biotin/avidin affinity chromatography. Several variations of this approach have been described including the solid phase ICAT [8] or the cleavable ICAT reagent [9,10]. Immobilised metal affinity chromatography (IMAC) is used for the selection of histidine-containing peptides [11–13] what is also a possibility for the selection of phosphorylated peptides [14–16]. This method uses the formation of chelate complexes with immobilised metal ions. Another way to select phosphorylated peptides is by elimination of the phosphate group and the attachment of an affinity moiety like biotin [17–20]. Recently, a labelling method for tryptophan residues has been presented using the increased hydrophobicity of labelled peptides for selection of the peptides [21]. Lectin columns are widely used for the selection of glycoproteins and peptides according to their glycosylation patterns [22–25]. Another approach directed to glycoproteins is based on hydrazide chemistry, where the peptides are reacted via the carbohydrate residues with immobilised hydrazide groups after oxidation of the *cis*-diol groups to aldehydes [26].

Histidine, cysteine and tryptophan are low-abundant amino acids. Selecting peptides with not so frequent amino acids might be an advantage because the complexity of tryptic mixtures can be significantly reduced. However, for proteins without these amino acids other methods have to be applied. Additionally, by selecting rare amino acids it is not always sure that a protein can be reliably identified if just one or two peptides are left for analysis after the enrichment step. Therefore, it might be advantageous in some cases to select peptides containing more frequent amino acids like for example arginine because then a better sequence and also proteome coverage should be achievable. The enzymatic digestion of proteins with trypsin, the most widely used proteolytic enzyme in protein analysis, leads to arginine and lysine terminated peptides. This makes arginine a potentially interesting target for affinity selection, although no such method has been described so far.

Boronate affinity chromatography is used since many years in the field of biomolecule purification [27]. Boronic acid groups are known to form a pH-dependent complex with the *cis*- and vicinal-diol groups of carbohydrates and polyphenolic compounds. The interaction is not limited to carbohydrates but to compounds containing *cis*-diol groups in general. Therefore, boronate chromatography has also been applied to the purification and enrichment of many compound classes like glycoproteins [28–30], nucleotides [31,32] and catechol compounds [33].

The reaction of the guanidine group of arginine with α-dicarbonyl compounds in the presence of borate buffer resulting in an adduct with an 1,2-diol function is known since the 1960s [34]. In this time there has already been an attempt to combine modification of arginine residues in proteins with 1,2-cyclohexanedione and boronate chromatography [35,36] but since then there has been no further effort to use this concept for the enrichment of Arg-containing peptides.

Recently, a selective tagging procedure for arginine residues in peptides and proteins in combination with mass spectrometry has been developed in our group [37]. This tag is based on the covalent modification of the guanidine group of arginine with 2,3-butanedione and an arylboronic acid (usually phenylboronic acid) under alkaline conditions (Fig. 1).

In this work an affinity selection technique directed to arginine based on this reaction is presented. Via the above-mentioned reaction the arginine peptides are trapped on a polymeric material with immobilised phenylboronic acid and separation from arginine-free peptides is possible. The solid-phase trapping procedure was optimised with a set of model peptides and the optimised method was then applied to tryptic digests of a mixture of three standard proteins (BSA, ovalbumin, lysozyme) to test the suitability for the application to more complex mixtures.

2. Materials and methods

2.1. Materials

All peptides were purchased from Bachem (Weil am Rhein, Germany) and lysozyme (hen egg-white), bovine serum albumin, ovalbumin and trypsin (from porcine pancreas, sequencing grade, E.C. 3.4.21.4) were from Sigma (Steinheim, Germany). The acrylic and agarose beads containing immobilised *m*-aminophenylboronic acid were supplied from Sigma (Steinheim, Germany) and an additional acrylic material was a kind gift of Prof. K.S. Boos. Phenylboronic acid (PBA) and deuterated (d_5) phenylboronic acid were from Aldrich (Steinheim, Germany). Butanedione (BD) was bought from Fluka (Buchs, Switzerland) and deuterated (d_6) butanedione from CQN Quality Stable Isotopes (Pointe-Claire, Canada). Usually water was double distilled prior to use, for nanoLC–MS/MS measurements gradient grade water from Fluka (Buchs, Switzerland) was used. All other chemicals and solvents were from Fluka, Riedel-deHaen (Seelze, Germany) or Fisher Chemicals (Loughborough, UK).

2.2. Binding protocol

Typically 500 μl of peptide solution (10 μg ml^{-1} of each peptide) were diluted with 500 μl of an ammonium acetate (NH_4Ac) solution (10–50 mM) with the pH adjusted to 10.5 with ammonium hydroxide and 40 μl of a 100 mM aqueous BD solution (approx. 100-fold molar excess). This solution was added to 200 μl of a suspension of agarose beads or 20 mg acrylic beads swollen in water (this corresponds to an approximately 100-fold molar excess of PBA-groups) and vortexed for 30–90 min.

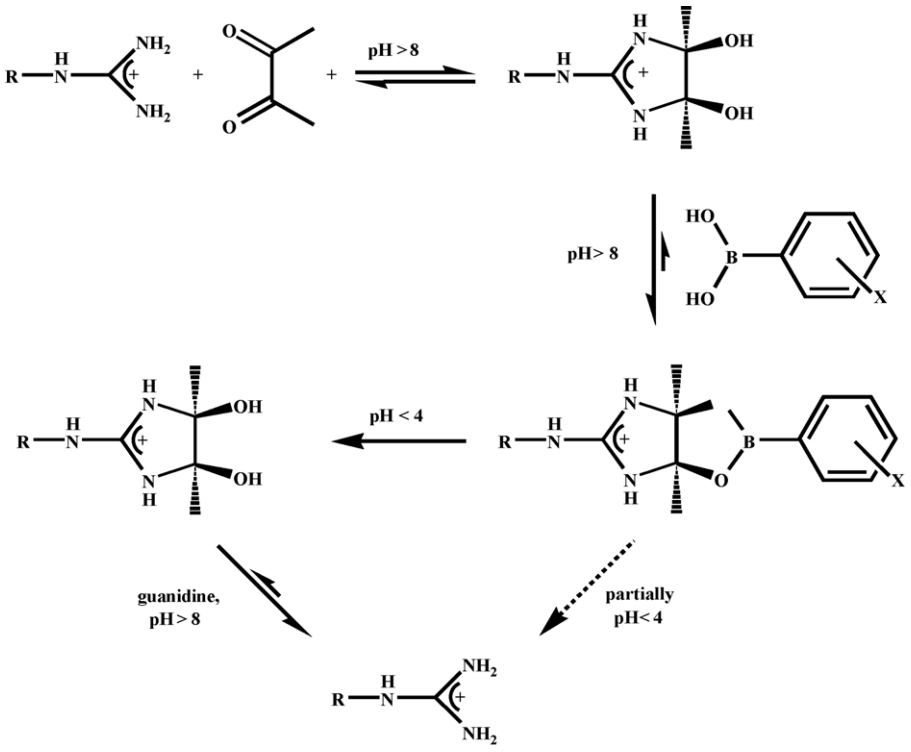

Fig. 1. Reaction principle of the solid phase capture and release method directed towards arginine-peptides using 2,3-butanedione and immobilised phenylboronic acid (X = anchor group).

After short centrifugation to settle the beads the supernatant was removed and the beads were washed twice with 500 μl of the washing solution by vortexing for 15–30 min. The washing solution consisted of NH_4Ac (10–50 mM) pH 10.5, water and acetonitrile in a ratio of 25:65:10 (v/v/v) containing both butylamine (approx. 4 mM) and butanedione in 50-fold molar excess. For elution of the Arg-peptides from the beads they were vortexed respectively for 15–30 min with 500 μl of elution solution consisting of water and acetonitrile 50:50 (v/v) containing 0.05% trifluoroacetic acid (TFA).

For cleavage of the dihydroxyimidazoline ring (see Fig. 1) an aliquot of the elution solution was dried under nitrogen. The residue was re-dissolved in 500 μl of a 1 mM aqueous guanidine hydrochloride solution, pH adjusted to 10 with ammonium hydroxide, and left for approximately 30–60 min. For LC–MS measurement the solution was acidified and spiked with 5 μg Lys-Bradykinin (Lys-BK) as internal standard.

Note: It is important that the second cleavage step with guanidine is performed in a plastic but not in a glass vessel as with those complete cleavage of the imidazoline ring could not be achieved.

2.3. Derivatisation and cleavage of peptides in solution

500 μl of a 0.1 mg ml^{-1} peptide solution were mixed with 500 μl of a 10 mM ammonium acetate solution adjusted to pH 10 with aqueous ammonia. To start the reaction 160 μl of a 50 mM solution of PBA and 40 μl of a 100 mM solution of BD were added and the solution was left at room temperature (RT) for approximately 90 min. For cleavage experiments of the covalent derivatives the reaction mixture was then acidified with the corresponding acid to adjust the desired pH value and left for approximately 60 min.

To investigate the label cleavage under acidic conditions d_5-PBA was used for derivatisation because the mass difference of an attached PBA and an attached BD for peptides containing two arginine residues is the same and cleavage of one whole tag and two boronic esters would not be differentiable. Using deuterated PBA leads to a mass shift of 2.5 amu for doubly charged peptides.

2.4. Tryptic digests

For the preparation of a tryptic digest of the model proteins equal amounts of the proteins (BSA, lysozyme, ovalbumin) were mixed and dissolved in 7 M urea solution. Disulfide bonds were reduced with DTT and cysteine groups were alkylated with iodoacetamide according to standard protocols. The sample solution was diluted 1:10 with 50 mM aqueous ammonium bicarbonate solution and tryptic digestion was performed for 16 h at 37 °C with an enzyme-to-protein ratio of 1:50 (v/v). The digest solution was then acidified and purified by solid phase extraction using reversed phase C18-cartridges.

For sample preparation with the PBA beads 30 μl of protein digest (1 mg ml^{-1} total protein) were applied to the beads using a 50 mM NH_4Ac solution following the procedure

described above. The acidic elution fractions were combined and aliquots were dried under nitrogen. The residue was redissolved in 50 μl of a 10 mM guanidine hydrochloride solution (pH 10) and left at RT for approximately 60 min. Prior to nanoLC–MS/MS analysis the solution was acidified with TFA.

2.5. LC–MS analysis

2.5.1. LC–MS analysis of peptide test mixtures

Liquid chromatography–mass spectrometry was performed on an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a PESciex API 365 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a standard electrospray source.

Model peptides were separated on a ThermoHypersil BetaBasic 18 column (150 mm × 4.6 mm I.D., Thermo Hypersil-Keystone, Bellefonte, PA, USA), equipped with a guard column of the same material. Gradient elution was performed at 25 °C using the mobile phases A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile. The gradient was from 5% B to 95% B in 25 min and the flow rate was 500 μl min^{-1}. Before the LC eluent was introduced in the mass spectrometer, the solvent was split in a ratio of approximately 1:100 by a restriction capillary connected to a mixing tee.

MS detection was performed in the single ion monitoring mode or by scanning an appropriate *m*/*z* range (for label cleavage experiments). Direct infusion experiments for optimisation of instrument settings and investigations on label cleavage were performed using a Harvard syringe pump (Harvard Apparatus, So. Natick, MA, USA) at a flow rate of 5 μl min^{-1}.

2.5.2. NanoLC–MS/MS analysis of the tryptic digests

Nanoflow liquid chromatography–tandem mass spectrometry was performed on an Agilent Nanoflow Proteomics Solution system consisting of an Agilent 1100 Series nanoLC system and an Agilent 1100 MSD Trap SL quadrupole ion trap mass spectrometer equipped with an orthogonal nanoelectrospray source.

First, 1 μl of the protein digest solution was injected onto an Agilent Zorbax 300 SB-C18 trapping column (5 mm × 300 μm I.D.) and the sample was washed for five minutes using 0.05% formic acid in water at a flow rate of 30 μl min^{-1}. Then, the trapping column was switched in-line with an Agilent Zorbax 300 SB-C18 column (50 mm × 75 μm). Gradient elution was performed using the mobile phases A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile. The gradient was as follows: 0–30 min from 5% B to 50% B, 30–35 min from 50% B to 80% B, 35–40 min 80% B. The flow rate was set to 250 nl min^{-1}. MS/MS detection was performed in the data-dependent mode, choosing the two most abundant ions from each full scan in the mass range of 400–1500 *m*/*z*. Dynamic exclusion was used for 2 min after the acquisition of two MS/MS spectra. Tandem MS spectra were acquired in the mass range of 300–2000 *m*/*z*.

MS/MS spectra were searched against the SwissProt database (dated 02/04/2004), using an in-house licence of MASCOT, version 2.0.5 (http://www.matrixscience.com), running on a 2.8 GHz Pentium 4 PC system with 2 GB of RAM. Parameter settings were as follows: taxonomy = chordata, enzyme = trypsin, up to one missed cleavage, carbamidomethylation on Cys as variable modification, charge states = 1+/2+/3+, instrument = ESI-Trap.

3. Results and discussion

3.1. Principle of the approach

In our group, a method for the covalent modification of the guanidine group of arginine with 2,3-butanedione (BD) and phenylboronic acid (PBA) under alkaline conditions has recently been developed (see Fig. 1) [37]. In this two step (but 'one pot') reaction 2,3-butanedione forms a *cis*-dihydroxyimidazoline ring with the guanidine group, which then is further condensed with phenylboronic acid to give a bicyclic system. The reaction was found to be specific towards arginine residues leaving other amino acids, like for example lysine and the N-terminus of peptides, unaffected. In this work the stability of the label under alkaline conditions was also investigated. The observed reversibility of the tagging reaction opens the possibility to use the concept for developing a solid phase enrichment technique depicted in Fig. 2.

First, the sample solution is adjusted to an alkaline pH (about 9–10) and butanedione is added. The mixture is applied to a polymeric material with immobilised phenylboronic acid and analytes containing a guanidine group (in our case the Arg-containing peptides) should be covalently bound to the solid support. By a selective washing step non-specifically bound Arg-free peptides (Lys-peptides in the case of a tryptic digest) can be removed without affecting the Arg-peptides covalently bound in form of the boronic acid complex. Elution of the Arg-peptides is performed by a pH switch to acidic conditions where the boronic ester bond is known to be cleaved. This way, the Arg-containing and Arg-free peptides should be separated with the binding and washing fractions containing the Arg-free peptides and the elution fractions containing only Arg-peptides.

3.2. Optimisation of the procedure

For optimisation of the procedure four Arg- and four Arg-free, but Lys-containing peptides were used as model analytes (peptides 1–8 in Table 1). They were chosen to consist of 6–13 amino acids having either Lys or Arg on the C-terminus (imitating a tryptic digest) with neurotensin, where the arginine residues are in the middle of the

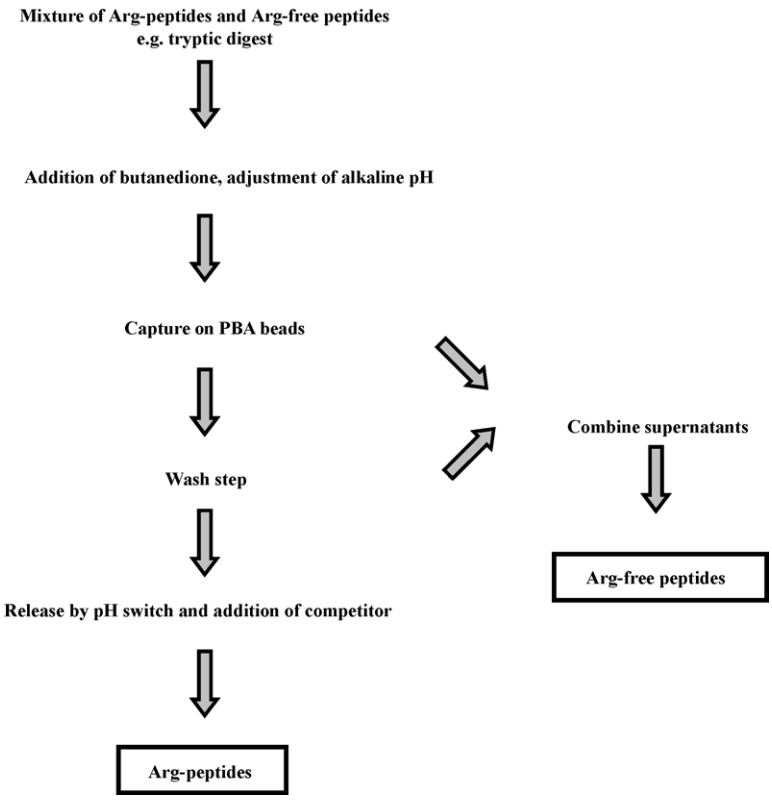

Fig. 2. Principle of the Arg-capture strategy. For details of the procedure, see text.

sequence, as an exception. Several polymeric materials with immobilised phenylboronic acid are commercially available. In this study three different kinds of materials with immobilised *m*-aminophenylboronic acid groups were used: two commercial materials, one based on poly(methacrylamide) (acrylic 1, 320 μmol PBA-groups/g dry beads) and one on agarose (50 μmol PBA-groups/ml) and additionally a non-commercial material also based on poly(methacrylamide) (acrylic 2, 400 μmol PBA-groups/g dry beads) with a slightly higher loading of phenylboronic acid.

Table 1
List of peptides used for the optimisation steps of the capture/release procedures with Arg- and Lys-residues marked in bold, molecular weight (monoisotopic form), *z* as the dominant charge state and mass to charge ratios used for MS detection

No.	Sequence (common name)	MW	*z*	*m*/*z*
1	PyrLYEN**K** (Neurotensin 1–6)	776.4	1+	777.4
2	VAITVL**K** (Ca-like peptide)	841.6	2+	421.8
3	YGGFM**KK** (Met-Enkephaline-LysLys)	829.4	2+	415.7
4	PHPFHFFVY**K**	1317.6	2+	659.8
5	PyrLYENKP**RR**PYIL (Neurotensin)	1673.0	2+	837.0
6	PPGFSPF**R** (des-Arg^1-Bradykinin)	903.5	2+	452.7
7	K**R**PAGFSPF**R** (Lys-Ala^3-Bradykinin)	1161.6	2+	581.8
8	**R**PPGFSPF**R** (Bradykinin)	1059.6	2+	530.8
9	K**R**PPGFSPF**R** (Lys-Bradykinin)	1187.7	2+	594.8
10	**R**PPGFSPF (des-Arg^9-Bradykinin)	903.5	2+	452.7
11	K**R**PPGFSPF (Lys-des-Arg^9-Bradykinin)	1031.6	2+	516.8

Preliminary experiments showed that it is possible to trap the Arg-peptides quantitatively on the solid support but that also some of the Lys-peptides are retained to a certain extent due to non-specific interactions. The amount was found to be strongly dependent on the peptide (sequence), varying in the range from 10 to 90%. This non-specific binding might be caused either by interactions with the material itself or by interactions of the boronate functionality with the Lys side-chain.

These results made the introduction of a selective washing step necessary to remove the non-specifically bound Lys-peptides so that a separation of Arg-containing and Arg-free peptides can indeed be achieved. The effect of alkylamine-type competitive washing additives (e.g. dibutylamine, butylamine, etc.), chosen to mimic the Lys side chain, was therefore investigated. Respective results obtained wit the acrylic material 1 are shown in Table 2 whereby an approximately 50-fold molar excess of butylamine was found to be a most suitable washing additive. The amount of retained Lys-peptides could be decreased to less than 60% for PHPFFFVYK and less than 20% for the others.

To achieve further improvement of selective washing steps, the influence of an organic modifier in the washing solution was tested. Various amounts of acetonitrile (10–20%) in the butylamine solution turned out to be beneficial as summarised in Table 2. An acetonitrile amount of 10% was found to be a good compromise between removing more of the Lys-peptides and not losing too much of the Arg-peptides.

Table 2
Comparison of the effect of a washing additive and an organic modifier on the amount of Lys-peptides retained obtained with the acrylic material 1

	PyrLYENK		VAITVLK		YGGFMKK		PHPFHFFVYK	
	Binding and washing	Elution	Binding and washing	Elution	Binding and washing	Elution	Binding and washing	Elution
Washing additive								
No additive	99.9	0.1	64.8	35.2	44.0	56.0	13.6	86.4
Alkylamine	99.6	0.4	94.3	5.7	84.3	15.7	41.4	58.6
Organic modifier								
10% CH_3CN	99.8	0.2	95.3	4.7	95.6	4.4	66.4	33.6
20% CH_3CN	99.9	0.1	98.3	1.7	96.9	3.1	79.5	20.5

The relative amounts (in %) of the four Lys-peptides in the binding and washing solutions and the elution fractions are shown. Washing conditions were without additive or using butylamine as competitor and competitor plus acetonitrile as organic modifier.

Additionally, variations of pH and ionic strength of the binding solution were investigated. A higher pH of about 10.5 turned out to be favourable to partially suppress binding of Lys-peptides. This should reduce the ionic interactions between the boronic acid functionality and the Lys side-chain by a partial deprotonation of the Lys-residue without affecting the ionisation of the Arg side-chain. Furthermore, using a binding solution with 50 mM ammonium acetate instead of 10 mM was found to reduce non-specific interactions of the peptides and also a positive effect on recovery of peptides was observed.

3.3. Cleavage from the beads

Cleavage of the bound Arg-peptides from the PBA-beads was performed under acidic conditions at a pH of approximately 2. Usually this was done with 0.05% TFA solution in water/acetonitrile in a ratio of 50/50 (v/v) in four fractions as optimised conditions.

The idea was to remove the complete label by this pH change but unsatisfactory recovery rates in the first experiments indicated that this was not so easily possible. On the search for the reasons we found that an incomplete cleavage of the dihydroxyimidazoline ring occurred under the applied conditions whereas hydrolysis of the cyclic boronic ester, to cleave the Arg-peptides from the solid support, proceeds completely and very fast. This results in a mixture of partially tagged arginine (still containing the dihydroxyimidazoline group derived from the BD addition) and the free guanidine group leading to a rather more complex than simplified mixture.

To study the cleavage of the label in more detail, the effect of different acids and pH values was investigated performing both the labelling reaction and cleavage in solution using six bradykinins as analytes (peptides 6–11 in Table 1). TFA, formic acid and acetic acid at pH 2–4 were tested.

The investigated peptides were derivatised with butanedione and phenylboronic acid in solution. After cleavage by addition of the corresponding acid the sample was measured directly by ESI-MS. For comparison, the relative intensities of the free and partially butanedione-derivatised peptides were calculated assuming that the ionisation efficiency for the free and the partially labelled forms are comparable [37].

Generally, no significant change in the cleavage rate was observed for the three acids and also for pH 2 and 3. But we found that obviously a pH lower than 4 is necessary for fast hydrolysis of the boronic ester.

In Table 3 the results for the different peptides obtained with TFA (conditions applied for cleavage from the beads) are summarised, showing that the rate of cleavage of the imidazoline ring is dependent on the peptide. Whereas for Lys-BK and Lys-Ala^3-BK more than 50% are cleaved, it is just around 20–30% for des-Arg^1-BK and des-Arg^9-BK. The cleavage rates for the other peptides are within this range.

In order to find conditions where complete cleavage of the label can be obtained some experiments on the label stability at different pH values were done. Comparative labelling studies using deuterated butanedione (d_6-BD) were performed in acidic (conditions for cleavage from the beads) and alkaline milieu (conditions for the labelling reaction) with Lys-Ala^3-BK and des-Arg^9-BK as analytes.

First, the stability of the partial label (imidazoline ring) was investigated under acidic conditions. Peptides labelled with d_0-BD were incubated with excess of d_6-BD in acidic solution (pH approx. 2). Additionally, the exchange of butanedione under alkaline conditions (pH approx. 10) was examined. Lys-Ala^3-BK, a peptide containing two arginines, was derivatised with d_0-BD and d_6-BD and PBA in two separate batches in the same concentration. After complete derivatisation the two solutions were mixed 1:1 and left at RT. The time course of the exchange was followed by mass

Table 3
Comparison of the cleavage ratios (in %) of the free peptides and those partially derivatised with one and two butanedione groups, using TFA for label cleavage

	Free	+1BD	+2BD
RPPGFSPFR	32	32	36
KRPPGFSPFR	44	30	26
KRPAGFSPFR	50	16	34
PPGFSPFR	21	79	–
RPPGFSPF	29	71	–
KRPPGFSPF	38	62	–

The derivatisation and the cleavage steps were performed in solution.

spectrometry. For both peptides and also for the reciprocal experiments absolutely no exchange of the partial butanedione label was observed under acidic conditions. In alkaline milieu a mixed derivative (containing one d_0-BD and one d_6-BD) occurred revealing that an exchange of BD within the whole label takes place but it was found to be very slow.

Due to the fact that the BD label is reversible under alkaline but in some cases not under acidic conditions we examined if the bound butanedione can be released also in alkaline milieu. The peptides labelled with BD were dissolved in alkaline ammonium acetate solution (pH 9–10) and left to rest at RT. It was found that after removal of the reagents (PBA and BD) the partially existing imidazoline ring is indeed slowly released. The kinetics of this reaction can be relatively slow, but after maximum of 24 h the cleavage was complete.

Accordingly it should be possible to accelerate cleavage of the BD label by the addition of a suitable competitor also at alkaline pH. In this case guanidine was used but generally any guanidine- or amidine-containing compound should be suitable as competitor. The six bradykinin analogues listed in Table 1 were used for these experiments. After elution from the beads under acidic conditions guanidine was added and the pH was adjusted to approximately 10. LC–MS analysis showed that using guanidine as competitor a cleavage rate of more than 95% is achieved within 30–60 min at RT under alkaline conditions (illustrated in Fig. 3).

3.4. Comparison of the materials

Three different polymeric materials with immobilised *m*-aminophenylboronic acid were tested using the optimised conditions developed for the eight model peptides. The two materials acrylic 1 and acrylic 2, based on poly(methacrylamide) and the agarose material were compared (results see Table 4).

The biggest difference in the properties of these materials was found in the extent of the non-specific interactions of the Lys-peptides. We found that the agarose based material shows a lower degree of non-specific interactions with the Arg-free peptides. Especially for the peptide PHPFHFFVYK the retained amount could be reduced from about 30% for the acrylic beads to less then 5% for the agarose beads. For the other peptides slightly improved results were obtained with the agarose material as well. These results show that a major part of the non-specific interactions is obviously caused by the polymeric material itself although it is difficult to say of which nature these interactions are. Of course, agarose has a less hydrophobic backbone than polyacrylic materials.

On the other hand, with the acrylic beads 2 better binding of Arg-peptides was obtained. This might be caused by a higher loading of PBA compared to the acrylic material 1 but binding was even better than with the agarose beads.

In a next step the method was applied to a tryptic digest of BSA using the agarose based PBA-beads (data not

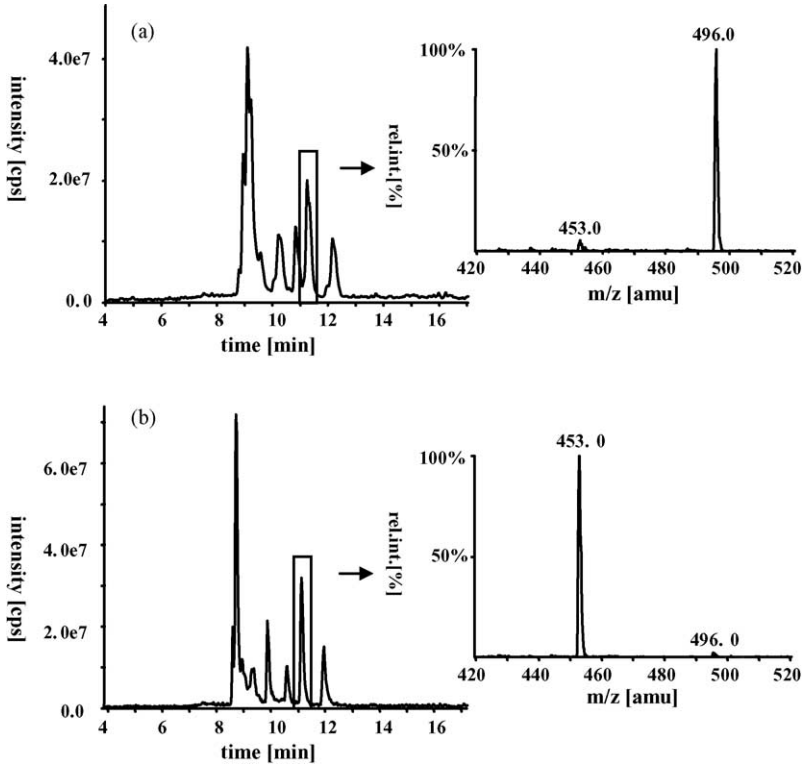

Fig. 3. Cleavage of six dihydroxyimidazoline ring labelled bradykinin analogues (6–11 in Table 1). The total ion chromatograms (a) after elution from the beads and (b) after cleavage with guanidine under alkaline conditions are shown with the corresponding mass spectra of des-Arg^1-BK with $m/z = 453$ for the free peptide and $m/z = 496$ for the partially tagged peptide (still containing the imidazoline-ring).

Table 4
Comparison of the results for the separation of the eight model peptides obtained with the three PBA-beads materials

	Agarose		Acrylic 1[a]		Acrylic 2	
	Binding and washing	Elution	Binding and washing	Elution	Binding and washing	Elution
PyrLYENK	99.6	0.4	99.8	0.2	99.6	0.4
VAITVLVK	93.1	6.9	95.3	4.7	92.0	8.0
YGGFMKK	97.3	2.7	95.6	4.4	85.5	14.5
PHPFHFFVYK	98.5	1.5	66.4	33.6	55.2	44.8
PyrLYENKPRRPYIL	0.3	99.7	4.2	95.8	0.1	99.9
PPGFSPFR	19.3	80.7	18.0	82.0	4.8	95.2
KRPAGFSPFR	0.0	100.0	6.3	93.7	0.0	100.0
RPPGFSPFR	0.1	99.9	0.2	99.8	0.1	99.9

The distribution of the peptides in the combined binding and washing fractions and the elution fractions are given relative to the total amount in %. The separation was performed according to the conditions described in Section 2 using a 50 mM NH_4Ac solution pH 10.5 and butylamine as washing additive.
[a] Experiments were performed using 10 mM NH_4Ac pH 10.5 as for this material no improvement was achieved with higher ionic strength.

shown). We found that a separation of Arg-containing and Arg-free peptides is generally possible. The Lys-peptides could be completely removed but problems with binding of some Arg-peptides was observed. Especially for acidic peptides (p*I* lower than 6) considerable amounts were found in the binding fraction together with the Lys-peptides. A possible reason that binding to PBA is not working well might be repulsion of like charges between the boronic acid functionality and the carboxyl groups of the acidic amino acid residues in vicinity to the guanidine group.

3.5. Optimised conditions

Applying the optimised conditions to the set of eight model peptides, a separation of Arg-containing and Arg-free, but Lys-containing peptides could be achieved satisfactory using the agarose material. Less than 5% of all of the Lys-peptides were retained unspecifically and maximum 10% of the Arg-peptides, most often less than 5%, were washed off from the beads during the washing step. Binding of acidic Arg-peptides caused some problems so these peptides were not completely bound to the PBA-beads. As noted above, the agarose based material showed less non-specific interactions of the Lys-peptides than the acrylic beads although binding of acidic peptides was found to work better with the acrylic PBA-material 2. With all PBA-materials, Lys-peptides were retained to some but minor extent, but this amount could be successfully reduced by the introduction of a thorough washing step. Cleavage of the Arg-peptides from the solid support was quantitatively achieved by a pH switch to acidic conditions, although to obtain the Arg-peptides in a free form, a second cleavage step was necessary to break apart the imidazoline ring completely. In alkaline milieu and by adding a guanidine-containing competitor more than 95% of the Arg-peptides are recovered in their native form as outlined in Fig. 3.

3.6. Application to tryptic digests

With this optimised procedure a separation of Arg-containing and Arg-free peptides should also be possible for tryptic digests considering the above described findings. On the one hand the agarose PBA-material showed a much lower degree of retention of Arg-free peptides than the acrylic PBA-material 2 but binding, especially of acidic peptides, was found to work better for the latter. The acrylic material 1 was excluded from these experiments because it showed the worst results for binding of Arg-peptides in general and especially the acidic ones. As compromise the agarose beads and the acrylic PBA-material 2 was used for the application to the protein digest.

A tryptic digest of a mixture of the three proteins BSA, ovalbumin and lysozyme in equal amounts was prepared. It was applied to the agarose and the acrylic PBA-beads. The washing and elution step were performed according to the procedure described above and the remaining imidazoline ring cleavage was performed using guanidine as competitor under alkaline (pH 10) reaction conditions. The affinity enriched peptides were then subjected to LC–MS/MS analysis and the obtained MS/MS data were database searched using MASCOT. The digest mix was measured both before affinity clean up and after performing the enrichment step for Arg-peptides. In Fig. 4 exemplary chromatograms, MS and MS/MS spectra of the measurements are shown. For evaluation of our method, the number of Arg-and Lys-containing peptides found by MASCOT search were compared. Peptides with a missed cleavage containing an arginine and a lysine residue were counted as arginine peptides, as all peptides containing an arginine residue should be bound to the PBA-beads.

In Table 5 the scores, sequence coverages and the number of Lys- and Arg-peptides found are listed. Generally, all three proteins were identified before and after the enrichment step. In the whole mixture 44 peptides could be identified, 23 Arg-peptides and 21 Lys-peptides. After applying the mixture to the PBA-beads the number of peptides was significantly reduced. With the acrylic material 19 peptides were recovered after the selection step, 18 Arg-containing and just 1 Lys-containing peptide. With the agarose beads 22 peptides (2 Lys-peptides and 20 Arg-peptides) were obtained. Besides 1 (acrylic) to 2 (agarose) Lys-peptides all other Arg-free peptides could be successfully removed from

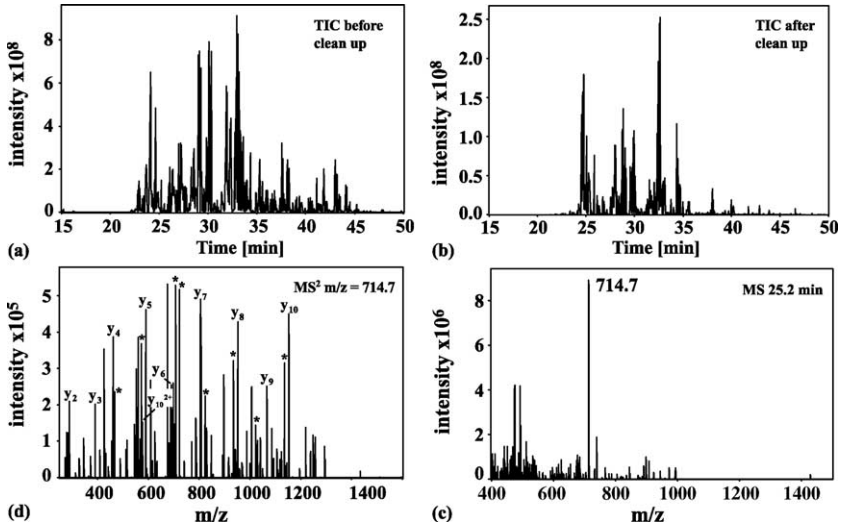

Fig. 4. NanoLC–MS/MS analysis of the tryptic digest. (a–b) Total ion chromatograms of the digest mixture before (a) and after (b) the arginine selection step. (c) Mass spectrum at 25.2 min with $m/z = 714.7$ as the doubly charged peptide FESNFNTQATNR from lysozyme and (d) MS/MS spectrum of the peptide FESNFNTQATNR, both from the analysis of the digest after affinity enrichment with the agarose beads. Signals marked with an asterisk correspond to b or y ions with additional loss of ammonia or water.

the mixture. Concerning the Arg-containing peptides 18 (acrylic) to 20 (agarose) out of 23 were could be retrieved after the selection step. The reduced number of peptide identifications might in part be explained by the fact that some peptides were present only at very low concentrations and might have been lost during clean-up. Ovalbumin for example is known to be relatively resistant towards proteolytic digestion which is already described in literature [38].

When the sequence coverages from Table 5 are compared, obviously lower values were obtained from the processed mixtures. The same applies to the MASCOT scores because only Arg-peptides are counted. However, special software could be designed to take into account that only Arg-peptides are present in the samples after clean-up. Whether this is a reasonable assumption will have to be inferred from further experiments.

The results show that selection of Arg-containing peptides from a tryptic digest is possible leading to a reduction of sample complexity (approx. 50%) while providing enough information for identification of the proteins. In this case identification of all three proteins is possible without further enrichment but real proteomic samples are usually much more complex. Generally, it should be possible to recover the Lys-peptides too (in the binding and washing fractions) but in this study this was not investigated. This and also the

Table 5
Results of the nanoLC–MS/MS analysis of tryptic digests of BSA, lysozyme and ovalbumin before and after performing the affinity capture step

	Before enrichment	After enrichment	
		Agarose beads	Acrylic beads
BSA			
No. Lys-peptides	14	–	–
No. Arg-peptides	9	7	8
MOWSE score	785	186	309
Sequence coverage (%)	51	15	18
Lysozyme			
No. Lys-peptides	3	1	–
No. Arg-peptides	7	7	6
MOWSE score	416	325	228
Sequence coverage (%)	82	72	39
Ovalbumin			
No. Lys-peptides	4	1	1
No. Arg-peptides	7	6	4
MOWSE score	408	230	180
Sequence coverage (%)	45	30	23

Two materials (PBA-agarose and the acrylic beads 2) were used. Conditions were as described in Section 2. Sequence coverage, MOWSE score and number of Lys- and Arg-peptides identified are compared.

application to complex samples will be a focus of our next studies.

Finally, chemo-affinity enrichment techniques are often combined with isotope labelling to enable relative quantitation of proteins from two different samples. In some cases the isotope label is directly incorporated into the introduced affinity tag, as for example in the ICAT method [7], or a global label directed to all peptides of the digest can be introduced (for example labelling of the N-termini) [39]. This strategy would also open the possibility for relative quantitation in combination with our Arg selective chemo-affinity enrichment technique in the context of matrix simplification.

4. Conclusions

A method for selective enrichment of Arg-containing peptides from peptide mixtures (e.g. tryptic digests) has been developed. It is based on a solid phase capture strategy trapping the Arg-peptides on a solid support with immobilised phenylboronic acid via a two step reaction. It should provide a reduction of sample complexity analysing complex protein mixtures. The selection of arginine as target, a relative frequent amino acid, should help to cover a wide range of proteins while still a simplification of the sample can be achieved.

The procedure was optimised concerning binding of the Arg-peptides, non-specific binding of Arg-free peptides and cleavage of the label after the selection step. Using the optimised protocol the method was applied to a tryptic digest of three proteins showing that the method can be also applied to real tryptic digests. It could be shown that it is possible to capture Arg-containing peptides from a mixture of tryptic peptides. All but one of the Lys-peptides could be completely removed from the mixture while nearly all Arg-peptides (20 of 23) were successfully recovered in high yields after the selection step.

Acknowledgements

The authors would like to acknowledge the financial support of the Christian Doppler Society (Vienna, Austria), Astra Zeneca R&D Mölndal (Mölndal, Sweden), Merck KGaA Darmstadt (Darmstadt, Germany) and piChem (Graz, Austria). Parts of the work were also supported by the Austrian Science Fund FWF (project # P15482). We would like to thank Prof. K.S. Boos for providing us the PBA-acrylic material.

References

[1] P.L. Ferguson, R.D. Smith, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 399.
[2] H.J. Issaq, T.P. Conrads, G.M. Janini, T.D. Veenstra, Electrophoresis 23 (2002) 3048.
[3] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[4] T. Wehr, PharmaGenomics 3 (2003) 36.
[5] F.E. Regnier, L. Riggs, R. Zhang, L. Xiong, P. Liu, A. Chakraborty, E. Seeley, C. Sioma, R.A. Thompson, J. Mass Spectrom. 37 (2002) 133.
[6] W.-C. Lee, K.H. Lee, Anal. Biochem. 324 (2004) 1.
[7] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[8] H. Zhou, J.A. Ranish, J.D. Watts, R. Aebersold, Nat. Biotechnol. 20 (2002) 512.
[9] K.C. Hansen, G. Scmitt-Ulms, R.J. Chalkley, J. Hirsch, M.A. Baldwin, A.L. Burlingame, Mol. Cell. Proteomics 2 (2003) 299.
[10] L.-R. Yu, T.P. Conrads, T. Uo, H.J. Issaq, R.S. Morrison, T.D. Veenstra, J. Proteome Res. 3 (2004) 469.
[11] A. Amini, A. Chakraborty, F.E. Regnier, J. Chromatogr. B 772 (2002) 35.
[12] D. Ren, N.A. Penner, B.E. Slentz, H. Mirzaei, F. Regnier, J. Proteome Res. 2 (2003) 321.
[13] D. Ren, N.A. Penner, B.E. Slentz, F.E. Regnier, J. Proteome Res. 3 (2004) 37.
[14] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 20 (2002) 301.
[15] P. Cao, J.T. Stults, Rapid Commun. Mass Spectrom. 14 (2000) 1600.
[16] C.S. Raska, C.E. Parker, Z. Dominski, W.F. Marzluff, G.L. Glish, R.M. Pope, C.H. Borchers, Anal. Chem. 74 (2002) 3429.
[17] M. Adamczyk, J.C. Gebler, J. Wu, Rapid Commun. Mass Spectrom. 15 (2001) 1481.
[18] Y. Oda, T. Nagasu, B.T. Chait, Nat. Biotechnol. 19 (2001) 379.
[19] M.B. Goshe, T.P. Conrads, E.A. Panisko, N.H. Angell, T.D. Veenstra, R.D. Smith, Anal. Chem. 73 (2001) 2578.
[20] W.-J. Qian, M.B. Goshe, D.G. Camp II, L.-R. Yu, K. Tang, R.D. Smith, Anal. Chem. 75 (2003) 5441.
[21] H. Kuyama, M. Watanabe, C. Toda, E. Ando, K. Tanaka, O. Nishimura, Rapid Commun. Mass Spectrom. 17 (2003) 1642.
[22] M. Geng, X. Zhang, M. Bina, F. Regnier, J. Chromatogr. B 752 (2001) 293.
[23] L. Xiong, D. Andrews, F. Regnier, J. Proteome Res. 2 (2003) 618.
[24] M. Geng, J. Ji, F.E. Regnier, J. Chromatogr. A 870 (2000) 295.
[25] H. Kaji, H. Saito, Y. Yamauchi, T. Shinkawa, M. Taoka, J. Hirabayashi, K.-I. Kasai, N. Takahashi, T. Isobe, Nat. Biotechnol. 21 (2003) 667.
[26] H. Zhang, X.-j. Li, D.B. Martin, R. Aebersold, Nat. Biotechnol. 21 (2003) 660.
[27] R.P. Singhal, S.S.M. DeSilva, Adv. Chromatogr. (New York, NY, United States) 31 (1992) 293.
[28] Y. Li, E.L. Larsson, H. Jungvid, I.Y. Galaev, B. Mattiasson, Bioseparation 9 (2000) 315.
[29] Y. Li, E.L. Larsson, H. Jungvid, I.Y. Galaev, B. Mattiasson, J. Chromatogr. A 909 (2001) 137.
[30] Y. Li, J.-O. Jeppsson, M. Jornten-Karlsson, E.L. Larsson, H. Jungvid, I.Y. Galaev, B. Mattiasson, J. Chromatogr. B 776 (2002) 149.
[31] B. Jerkovic, H.C. Kung, P.H. Bolton, Anal. Biochem. 255 (1998) 90.
[32] M. Rosenberg, J.L. Wiebers, P.T. Gilham, Biochemistry 11 (1972) 3623.
[33] A. Rudolphi, K.S. Boos, D. Seidel, Chromatographia 41 (1995) 645.
[34] J.F. Riordan, Mol. Cell. Biochem. 26 (1979) 71.
[35] K. Rose, J.D. Priddle, R.E. Offord, J. Chromatogr. 210 (1981) 301.
[36] L. Patthy, A. Varadi, J. Thesz, K. Kovacs, Eur. J. Biochem. 99 (1979) 309.
[37] A. Leitner, W. Lindner, J. Mass Spectrom. 38 (2003) 891.
[38] Y.-Q. Yu, M. Gilar, P.J. Lee, E.S.P. Bouvier, J.C. Gebler, Anal. Chem. 75 (2003) 6023.
[39] S. Julka, F. Regnier, J. Proteome Res. 3 (2004) 350.