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Short Communication

Facile detection of specific RNA-polypeptide interactions by MALDI-TOF mass spectrometry

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Abstract: A simple method for the detection of specific RNA-polypeptide interactions using MALDI-TOF mass spectroscopy is described. Instead of direct observation of the RNA-polypeptide complex, we attempted the indirect observation of the binding event by focusing on the disappearance of the free polypeptide signal upon interaction with RNA. As a result, specific binding of the Rev-response element (RRE) RNA of the HIV with two RRE-binding peptide aptamers, DLA and RLA peptides, as well as the bacteriophage λ boxB RNA with the λ N peptide was observed. We also show that specific RNA-binding peptides can be identified from a mixture of peptides with varying RNA-binding affinity, showing that the method could be applied to high-throughput screening from simple peptide libraries. The method described in this study provides a quick and simple method for detecting specific RNA–polypeptide interactions that avoids difficulties associated with direct observation of RNA and RNA–polypeptide complexes, which may find various applications in the analysis of RNA–polypeptide interactions and in the identification of novel RNA-binding polypeptides. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: RNA–polypeptide interactions; MALDI-TOF mass spectrometry; arginine-rich peptide; HIV RRE; lambda boxB; peptide library

INTRODUCTION

RNA–polypeptide interactions are intimately involved in various steps of gene regulation, and various methods for the detection and analysis of RNA–polypeptide interactions have been developed. However, *in vitro* methods such as calorimetry, gel mobility shift assays, surface plasmon resonance (SPR) require large quantities of substrate or radioactive/fluorescent labeling of either the RNA or polypeptide. Genetic methods for detecting RNA–polypeptide interactions such as the three-hybrid system [1] and the antitermination system [2] require cloning of sequences encoding the RNA and polypeptide of interest into plasmid vectors and yeast/bacterial culture. The development of simple methods for the detection of RNA–polypeptide complexes would be expected to facilitate the analysis of RNA–polypeptide interactions, and the identification of novel RNA-binding polypeptides.

The development of soft ionizing methods in mass spectrometry has made possible the simple and rapid detection of biological samples including RNA. MALDI-TOF mass spectrometry is the easiest and the most widely available method for the analysis of RNA [3]. However, in addition to the generally inefficient desorption of RNA, a number of difficulties that are common for oligonucleotides exist for the analysis of RNA. First, sample preparation is a key step in RNA analysis, as impurities can result in reduced sensitivity [4]. The choice of the proper combination of matrix material has been shown to be crucial for successful analysis, and 3-hydroxypicolinic acid (3-HPA) [5] and 2,4,6-trihydroxyacetophenone (2,4,6-THAP) [6] in combination with organic ammonium salts were found to be useful matrices. Organic ammonium salts such as ammonium citrate and ammonium tartrate were shown to suppress addition of alkali metals, which lead to the division of the molecular ion into multiple peaks ([M + Na] and [M + K]) and a reduction in resolution [6,7]. Ammonium salts have also been shown to suppress fragmentation of the oligonucleotides [7]. The 5′- and 3′-heterogeneity of T7 RNA transcripts has also been to implicated as a source of peak widening and low resolution [8]. Despite these advances, however, the sensitivity of RNA detection is much reduced compared to analysis of polypeptides of

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comparable size. While there has been a report that an enzymatically synthesized RNA of 461 nucleotides could be detected [8], there are few examples of the analysis of oligomers longer than 20 nucleotides.

In this study, the development of a novel method for the facile detection of specific RNA-polypeptide interactions by MALDI-TOF MS was attempted. While the detection of non-covalently linked complexes of short RNAs (9 to 19 nucleotides) and peptides (10 to 18 amino acids) has already been reported [9], simple and convenient analysis of such complexes was expected to be hampered by the difficulties associated with RNA as described above. We therefore focused on the disappearance of the signal for the more readily detectable polypeptide upon specific binding to RNA. In other words, it was expected that the signal for the RNA-binding peptide would not be detected when bound specifically to the RNA, while those peptides that do not bind specifically to the RNA would be observed, resulting in the indirect observation of specific RNA-polypeptide complex formation.

A 34-nucleotide RNA stem-loop from the Rev-response element (RRE) of HIV and RRE-binding peptides (DLA and RLA peptides) with differing affinities for the RRE [10], as well as the 19-mer boxB RNA from phage λ and the λ N peptide [11] were used as model RNA–polypeptide complexes (Figure 1). Using MALDI-TOF MS, it was shown, as expected, that specific RNA-binding by RRE-binding peptides and the λ N peptide could be observed by the disappearance of the peptide signal upon mixing with RNA. It was also shown that the identification of RNA-binding peptides from mixtures of peptides with differing RNA-binding affinity is possible.

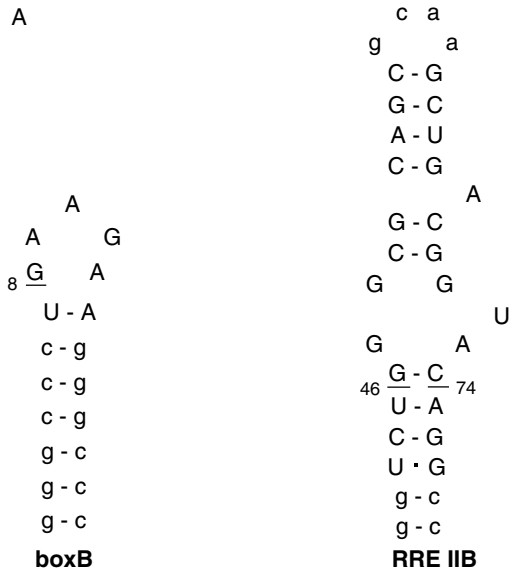

Figure 1 RNAs and RNA-binding peptide. (A) Secondary structures of the boxB RNA and the RRE IIB RNA. The underlined nucleotides are substituted in mutant RNAs. (B) RNA-binding peptides. The DLA and RLA peptides bind to RRE, the N peptide binds to boxB RNA. The underlined amino acids were not included in the synthetic peptide.

RESULTS AND DISCUSSION

Confirmation of RNA-peptide Interactions Using a Bacterial Reporter System

The interactions of the RNAs and peptides used in the present study were confirmed using a bacterial reporter system that utilizes the bacteriophage λ N protein mediated antitermination to monitor RNA–polypeptide interaction (Table 1) [2]. The pBR plasmids encoding the DLA, RLA or λ N peptides were transformed into pAC LacZ reporter cells encoding either the RRE or boxB RNA (Figure 1), and antitermination activity was assayed on tryptone plates containing X-gal. The intensity of colony color represented as plusses has been shown to correlate with binding affinity, where the interaction between the RRE RNA and the DLA peptide (Kd = 0.5 nM) reports an antitermination activity of 6+ [10], while that of the RRE and the RLA peptide (Kd = 30 nM) was 3.5+ [10], and that of the λ boxB RNA and the λ N peptide (Kd = 20 nM) was 8+ [11]. On

Table 1 The *in vivo* activities (X-gal assay) and Kd values (nM) of the DLA, RLA, λ N peptides with the boxB and RRE IIB

	X-gal (Kd, nM)[a]					
	DLA		RLA		λ N	
RRE	++++++	(0.5)	+++(+)	(30)	—	
RRE C46G74						
boxB	—		—		++++++++	(20)
boxB G8A						(>2560)

[a] The number of plusses indicates blue color scored by the colony color (X-gal) assay.

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the other hand, the interaction between the RRE and the λ N peptide, as well as the interaction between the boxB with both the DLA and RLA peptides did not show antiterrmination activity, and could not be detected.

Optimization of the Matrix Material for RNA and Polypeptide Detection

A number of different matrices, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 3-HPA, 2,3,4-THAP and 2,4,6-THAP, were tested for the analysis of the above mentioned RNAs, peptides and RNA–peptide complexes in both the negative and positive-ion mode. CHCA is widely used for the analysis of various materials [12], and DHB is used for DNA and sugar analysis [13].

All measurements were repeated three times and contained a synthetic peptide, P14R peptide (MW 1534) or angiotensin II (MW 1046), as an internal standard. Ammonium citrate was included to suppress addition of alkali metals. Only peaks of the peptides were observed clearly with CHCA, DHB and 2,4,6-THAP, and were similar in both ion modes. The signals of the RNAs were weak with all matrices, and the RNA–peptide complex, RRE–DLA complex, could only be detected with 2,4,6-THAP in positive-ion mode. Therefore, all of the following mass analyses were performed with 2,4,6-THAP in the positive-ion mode.

The Detection of RNA–polypeptide Complex Formation by the Disappearance of the Free-Peptide Signal

We tried to detect RNA–peptide complex formation by monitoring the decrease of the signal corresponding to the peptide, using the P14R peptide as an internal standard. When ammonium citrate and 2,4,6-THAP were added immediately after mixing the RRE RNA and the DLA peptide to form a solid solution, the peak of the free peptide was observed (Figure 2(A)). When ammonium citrate and 2,4,6-THAP were added after a 10-min incubation of the RNA and peptide at room temperature or on ice, followed by solid solution formation, the peak of the RNA-binding peptide was not detected (Figure 2(B)). In addition, the intensity of the peak corresponding to the DLA peptide decreased as the molar ratio of RRE RNA added was gradually increased in 0.25 molar equivalent increments (Figure S1, supporting data). This suggested that, as expected, upon binding of the DLA peptide with the RRE RNA, the DLA peptide was no longer released upon laser desorption, thereby providing a means to indirectly detect the RNA–peptide binding event. Therefore, all of the following mass analyses were performed after a ten-minute incubation of the mixture of RNA and peptide at room temperature.

The Detection of Specific RNA–peptide Interactions

First, the interaction of HIV RRE RNA and RRE-binding peptides was examined. When a mixture of the DLA peptide, which binds to RRE with high affinity and high specificity (Kd = 0.5 nM, Table 1), was analyzed, the DLA peptide showed a mass peak with an intensity comparable to that of P14R, the internal standard (Figure 3(A)). In comparison, upon addition of the RRE RNA to this mixture, a complete disappearance of the signal corresponding to the DLA peptide was observed, while the peak of the P14R peptide remained unchanged (Figure 3(B)), as also observed in Figure 2(B). On the other hand, when an RRE mutant (C46G74) that does not bind to the DLA peptide was added, no effect on the DLA signal was observed (Figure 3(C)). This suggested that the disappearance of the DLA peak upon addition of the wild-type RRE represented a specific binding event, and was not due to non-specific binding. Next, when a mixture of the RRE RNA and the RLA peptide, which binds to the RRE with a Kd of 30 nM (Table 1), was analyzed, the peak corresponding to the RLA peptide disappeared (Figure 3(D), (E)). Furthermore, as in the case of the DLA peptide described above, the signal corresponding

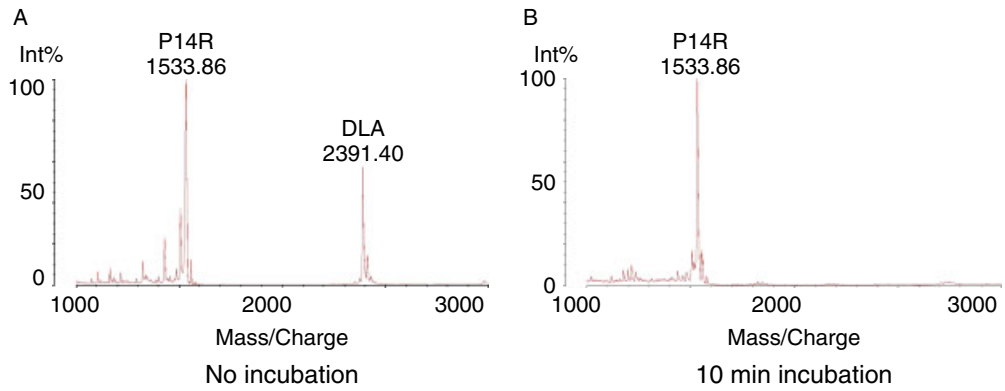

Figure 2 MALDI-TOF mass spectra of DLA peptide-RRE RNA. (A) No incubation (B) 10-min incubation. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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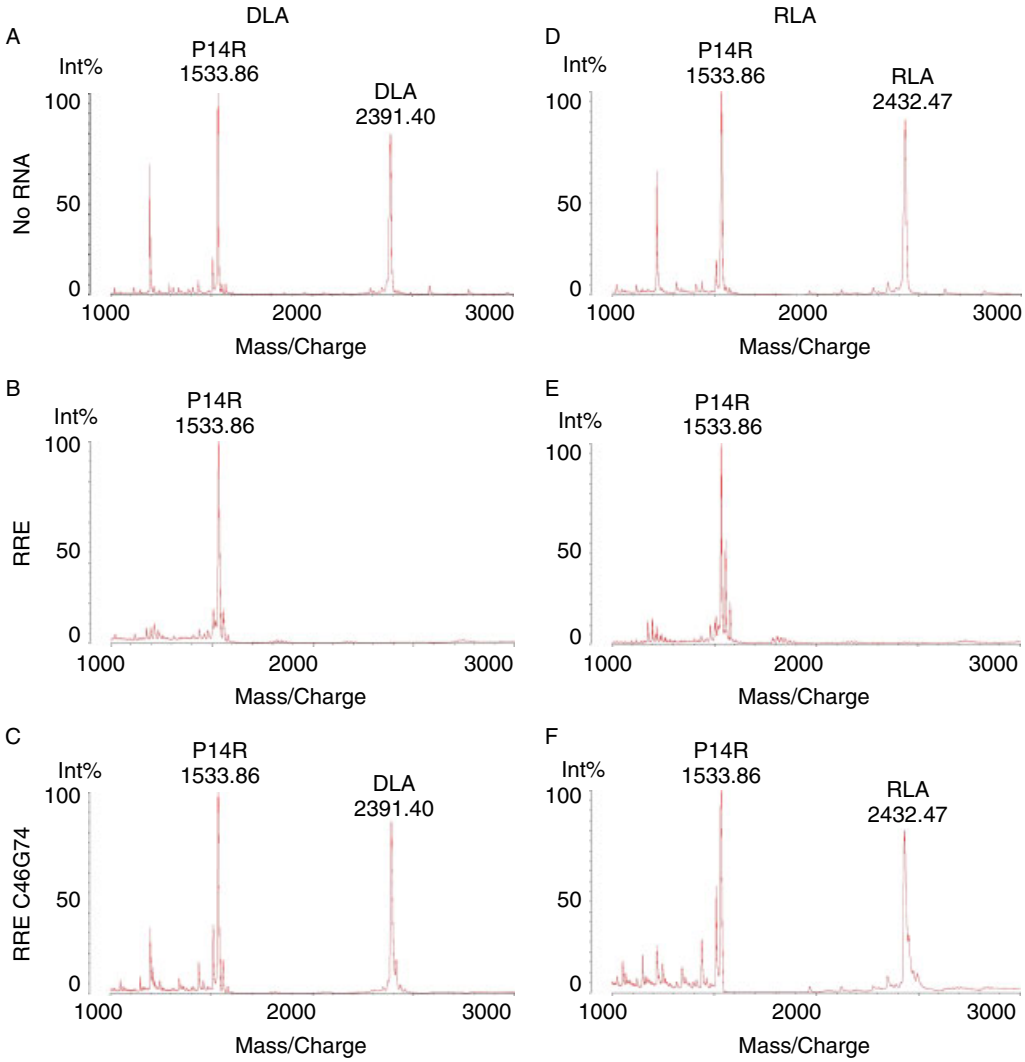

Figure 3 MALDI-TOF mass spectra of the free peptides and mixtures of the peptides and RRE RNA or RRE C46G74 RNA. (A) DLA peptide (B) DLA peptide-RRE RNA (C) DLA peptide-RRE C46G74 RNA (D) RLA peptide (E) RLA peptide-RRE RNA (F) RLA peptide-RRE C46G74 RNA. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

to the RLA peptide did not disappear upon addition of the RRE mutant (Figure 3(F)), showing that specific RNA–polypeptide interactions with a Kd of at least more than 30 nM could be analyzed by this method. As expected, addition of the wild-type and mutant RRE to the λ N peptide, which does not bind specifically to the RRE, resulted in no change in the peptide signal (Figure S2, supporting data).

In order to confirm that specific binding of the λ N peptide with the boxB RNA could also be observed by this method, the interaction of the DLA, and RLA, and λ N peptides with the wild-type boxB and a mutant boxB was analyzed (Figures 4 and S3, supporting data). Addition of the wild-type and mutant boxB RNAs to the DLA and RLA peptides resulted in the retention of the signals corresponding to the peptides (Figure S3(A)–(F), supporting data). On the other hand, addition of the wild-type box B to the λ N peptide resulted in an almost complete disappearance of the signal corresponding to the peptide, while the addition of the mutant boxB (G8A) did not affect the peptide signal (Figure 4). This further confirmed that specific RNA-polypeptide binding could be simply detected by whether or not the free-peptide signal in the MALDI-TOF MS vanishes upon RNA addition.

Identification of Specific RNA-binding Peptides from a Simple Peptide Library

In order to elucidate the utility of the present technique to identify RNA-binding peptides from mixtures of peptides with varying RNA-binding affinities, mixtures of peptides were treated with either the RRE or the RRE mutant (Figure 5). As a result, for example, when the DLA, RLA and λ N peptides were mixed with the RRE, only the signals corresponding to the DLA and RLA peptide disappeared (Figure 5(B)), while both the signals were present when the RRE mutant was used (Figure 5(C)).

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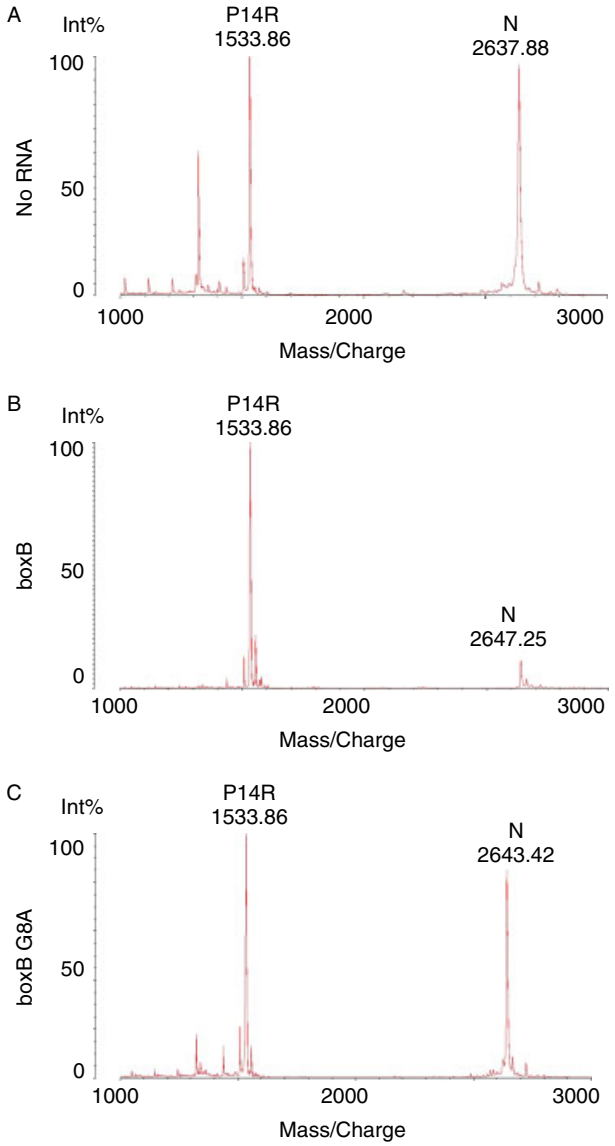

Figure 4 MALDI-TOF mass spectra of the free peptide and mixtures of the peptide and boxB RNA or boxB G8A RNA. (A) λ N peptide (B) λ N peptide-boxB RNA (C) λ N peptide-boxB G8A RNA. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

CONCLUSIONS

A simple method for detecting specific RNA–polypeptide interactions by MALDI-TOF MS that avoids difficulties associated with the direct analysis of the RNA-polypeptide complex, and is expected to be useful in the qualitative analysis of RNA-polypeptide interactions is described. The ability to identify strongly binding peptides from a mixture of similar peptides with varying RNA affinity, combined with the ease with which analysis can be carried out, suggests that MALDI-TOF MS may be applicable for the high-throughput screening of specific RNA-binder from simple peptide libraries.

MATERIALS AND METHODS

The Preparation of RNAs and Peptides

The RNAs were prepared by *in vitro* transcription using T7 RNA polymerase of synthetic oligonucleotide templates for RRE (5′-GGCCTGTACCGTCAGCTTGCGCTGCGCCCAGACCTATAG-TGAGTCGTATTAC-3′), RRE C46G74 (5′-GGCCTCTACCGTCA-GCTTGCGCTGCGCCCAGACCTATAGTGAGTCGTATTAC-3′), boxB (5′-GGGCCCTTCTTCAGGGCCCTATAGTGAGTCGTAT-TAC-3′), and boxB G8A (5′-GGGCCCTTCTTTAGGGCCCTATA-GTGAGTCGTATTAC-3′) that were annealed to the T7 primer (5′-GTAATACGACTCACTATA-3′). The RNA oligonucleotides were purified in denaturing PAGE, and desalted on a NAP 5 columns (Amersham Biosiences). The RNA was dissolved in H_2O to 100 pmol/μl, and were annealed by heating at 95 °C for 5 min and quick-cooling to 0 °C. The arginine-rich peptides were constructed on XX resin using automatic peptide synthesizer (Applied Biosystems, Model 433A) starting from Fmoc-Arg(pmc)-resin (0.25 mmol/g) with standard Fmoc-chemistry. The peptides were deprotected and cleaved from the resin by the treatment with TFA for 4 h at room temperature. The peptides were isolated and purified by HPLC with linear gradient conditions of acetonitrile/H2O/0.1% TFA as eluent. The peptides were lyophylized as fluffy white powder of the acetates and analyzed by MALDI-TOF MS using Voyger Linear II. The [M + H] cations were detected for all synthetic peptides. The λ N peptide used in this study was shorter by four amino acid residues at *C*-terminal than that used for gel shift (Figure 1). These amino acid residues are not important for binding to boxB RNA [14].

Mass Spectrometric Analysis

The matrices CHCA, DHB, 3-HPA were obtained from SIGMA-ALDRICH, and 2,4,6-THAP was obtained from Fluka. Ammonium citrate was obtained from SIGMA-ALDRICH.

One microliter of the RNA and 1 μl of the peptide (100 pmol/μl in H_2O), in the case of the mixtures of RRE or RRE C46G74 RNAs and all peptides, 3 μl of the RNA (100 pmol/μl) and 0.1 μl of the peptide (1 nmol/μl in H_2O) were incubated together in a 1.5-ml tube for 10 min at room temperature (20 °C) or on ice. Following the addition of 0.5 μl of 0.2 N ammonium citrate and 0.5 μl of ProteoMass P14R MALDI-MS Standard (SIGMA-ALDRICH), each sample was dropped on a sample plate. Then, the matrix solution 1.2 μl of 20 mg/ml 2,4,6-THAP in methanol, 1 μl of 15 mg/ml DHB in 0.1% TFA/acetonitrile (2 : 1) or 1.2 μl of 60 mg/ml 3-HPA in H_2O, was dropped on the sample very carefully, and dried. After drying the spots on the plate completely, the mass analysis performed with Axima CFRplus MALDI-TOF MS spectrometer (Shimadzu, Japan). Each spectrum was averaged for 100 laser shots in linear mode at 120 mV.

RNA-peptides Binding Assay *In Vivo*

pBR plasmid DNAs activities were monitored using the LacZ colony color assay [2] with N567/reporter cells containing pAC-RRE or pAC-λ nut plasmids. N567/pAC cells (50 μl) were transformed using 1 μl of the pBR plasmid DNA (10 ng) by heat shock, and incubated in the tryptone medium (0.5 ml) at 37 °C for 1 h. A portion of the culture was spread onto

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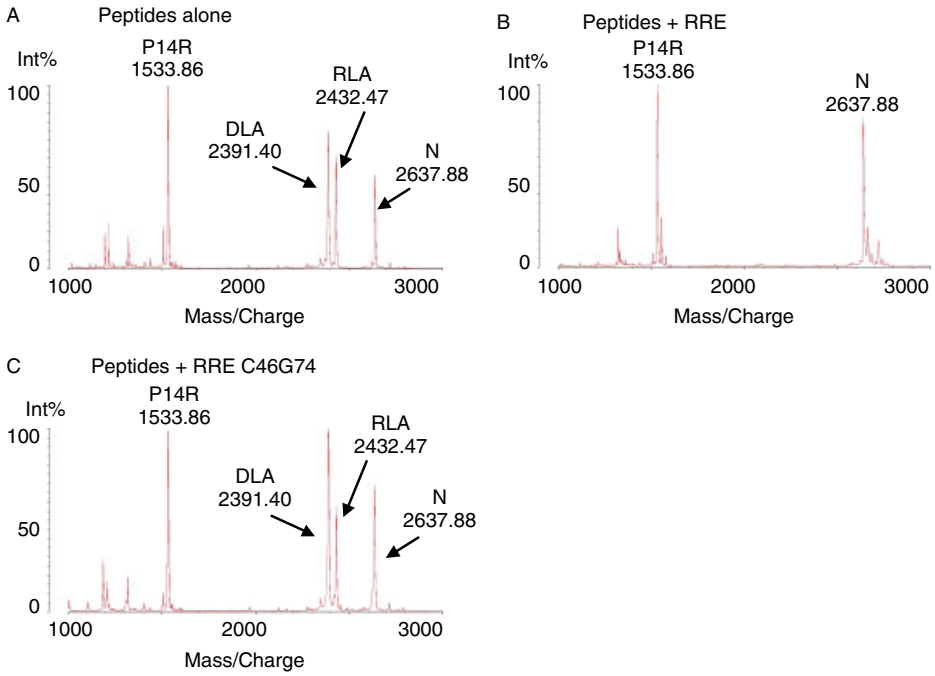

Figure 5 MALDI-TOF mass spectra of mixed peptides and RRE RNA or RRE C46G74 RNA. (A) DLA, RLA, and λ N peptides (B) DLA, RLA, and λ N peptides-RRE RNA (C) DLA, RLA, and λ N peptides-RRE C46G74 RNA. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

tryptone plates (60 mm diameter) containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 0.05 mM IPTG, 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside (X-gal), incubated at 37 °C for 24 h, and the proportion of blue colonies was scored [2].

Supplementary Material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

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