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An ESI Q-TOF study to understand the impact of arginine on CID MS/MS characteristics of polypeptides

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ABSTRACT

To understand the importance of arginine (Arg) in influencing electrospray ionization (ESI) - collision induced dissociation (CID) tandem mass spectrometry (MS/MS) behavior of peptides of lengths > ~ 25 amino acid residues (a.a.r.), we chose carbamidomethyl Insulin B-chain (30 a.a.r.), glucagon (29 a.a.r.) and melittin (26 a.a.r.) as the models for this investigation. Also, two smaller peptides: Angiotensin II (8 a.a.r.) and Bradykinin (9 a.a.r.) were studied for better comprehension of the interplay between the influence of Arg and peptide's length. The motivation to study such longer peptides stems from middle-down proteomics, a recently emerging field encompassing investigations on proteolytic peptides longer than ~ 25 a.a.r. CID MS/MS data of two different cases have been compared: (1) standard model peptides *vs.* chemically modified peptides, wherein sidechain guanidine group of Arg residues in these model peptides are selectively modified by 1,2-cyclohexanedione and phenylglyoxal; (2) standard model peptides *vs.* mutated model peptides, in which Arg residues in these model peptides are substituted by alanine (Ala) residues (Arg → Ala).

Different types of stoichiometric products were obtained due to this chemical modification and each type of arginine-modified product was subjected to ESI CID MS/MS. The Arg → Ala mutated model peptides chosen for this study are: [R22A]-Insulin B-chain, [R17A & R18A]-Glucagon, [R22A & R24A]-Melittin and a shorter peptide: [R2A]-Angiotensin II. All experiments were performed in a quadrupole time-of-flight hybrid mass spectrometer, whereby CID (hexapole collision cell) was done by following fixed collision energy (CE) as well as ramped CE. Analysis of CID MS/MS spectra revealed that the sequence coverage of Arg → Ala mutated peptides was higher than the chemically modified peptides as well as their respective standard (unmodified) peptides. Examination of all the MS/MS data alluded that Arg has a greater influence on the CID MS/MS behavior of longer peptides, viz., lengths > 25 a.a.r., than the smaller peptides.

1. Introduction

Tandem mass spectrometry (MS/MS) in conjunction with the two soft ionization modes, matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), not only constitutes major backbone for proteomics, but it also facilitates elucidation and ascertaining the molecular structure of various kinds of molecules. With regard to proteomics, among different approaches, middle-down approach has gained significant attention, recently [1]. Because this approach involves characterization of longer proteolytic peptides, it enables better identification of post translational modifications (PTMs) and deducing PTMs is important to understand several crucial biological processes [2]. ESI based MS/MS has proven to be more useful to detect PTMs (e.g., acetylation, phosphorylation, isoforms, etc.) through MD proteomic approach, which mainly involves higher energy collisionally activated dissociation (HCD), electron capture dissociation (ECD), electron transfer dissociation (ETD) and ETcaD; in fact, the electron-based MS/MS methods: ECD, ETD and ETcaD have contributed to a greater extent for PTM discovery [2–4]. The utility of only collision induced dissociation (CID) MS/MS has rather not found to be fruitful for MD approach, but it has been applied in conjunction with ETD, e.g., ETcaD, which in turn has helped for the advancement of MD approach based proteomics [5–9].

Nevertheless, CID MS/MS alone has proven to be very successful for bottom-up proteomic approach, whereby sequences of

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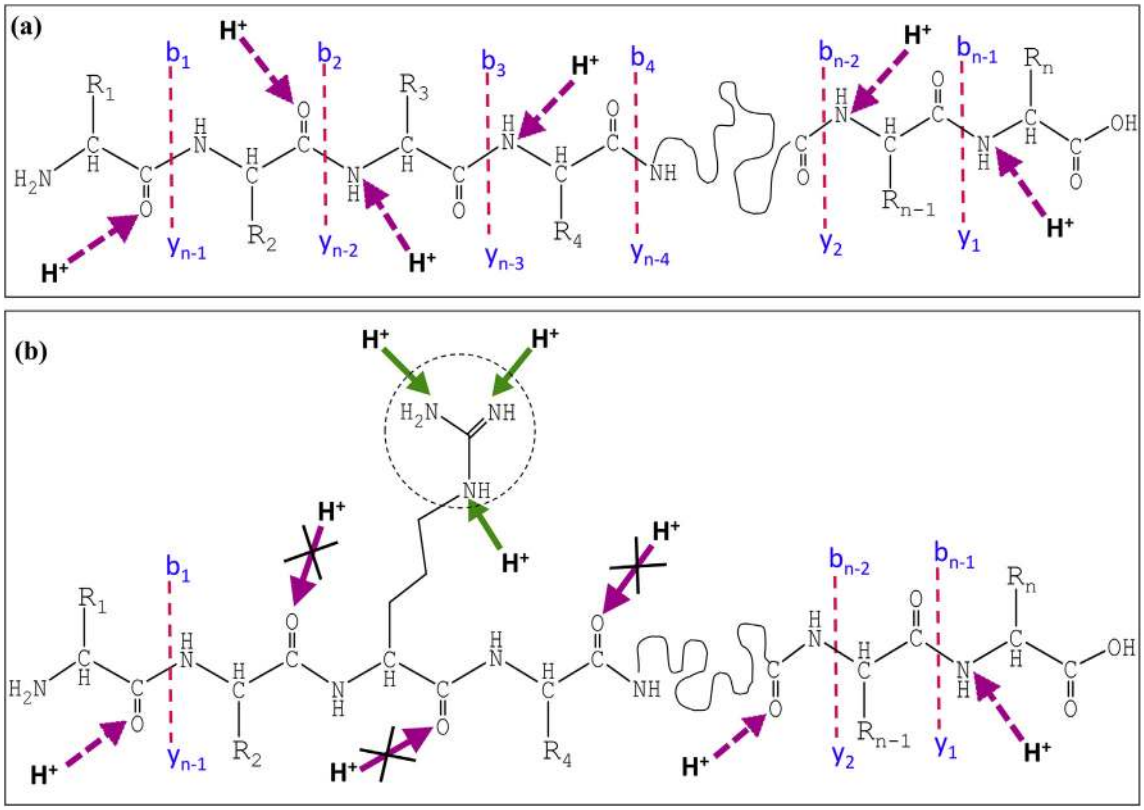
proteolytic (mainly tryptic) peptides that are not longer than about 20 amino acid residues (a.a.r.), can be unambiguously deduced from the CID MS/MS data of precursor ion charge states: $+1 \leq z \leq +3$; among which most of the peptide sequences have been deduced from the CID of precursor ion charge state, $z = +2$ [10]. Thus, in this study, our objective is to investigate CID MS/MS characteristics of peptides, which are longer than 25 a.a.r., with the focus on the precursor ion charge states, $+3 \leq z \leq +6$. It can be realized that this objective is commensurate with MD approach, since MD approach also involves MS/MS of peptides of lengths greater than 25 a.a.r., which adopt precursor charge states in the range, $+3 \leq z \leq +6$. So, this means that our interest is to explore, as to, what is/are the factor(s) that limit(s) the applicability of CID MS/MS for MD approach. Towards this objective, we have specifically chosen three model peptides of lengths in the range 25 - 30 a.a.r., whereby each peptide possesses at least one arginine (Arg) residue in it's sequence. This is because, arginines in the peptide sequence may not permit protonation of the backbone amide units, due to the presence of sidechain guanidine group, (which is a basic functional group) and consequently, can hamper the production of b- and y-type ions that result from the CID of the backbone amide units (Scheme 1). In other words, presence of arginine(s) in the peptide sequence can preclude the formation of contiguous series of b- and y-ions, thereby causing ambiguities in obtaining full sequence coverage. Hence, we hypothesize that the presence of Arg residues in the sequences of longer peptides may be a limiting factor, not permitting the applicability of CID MS/MS for MD approach.

To test this hypothesis, our another objective of this study is to assess the sequence coverage of the model peptides (as determined by CID MS/MS), upon chemically modifying the sidechain guanidine groups of arginines. For this chemical modification, we have employed phenylglyoxal (PG) and 1,2-cyclohexanedione (CHD), which are well known to covalently modify the sidechain guanidine group of arginine, in a very selective manner [11,12]. Further, we also wanted to examine the effect of replacing the Arg residues with alanine (Ala) residues (Arg → Ala) on the extent of sequence coverage of model peptides, as known from CID MS/MS. The standard model peptides selected for this investigation are: (i) Insulin B-chain (30 a.a.r.), whose sequence contains 1 Arg; (ii) Glucagon (29 a.a.r.), which possesses 2 Args in it's sequence; (iii) Melittin (26 a.a.r.), which also has 2 Args in it's sequence, and the 'Arg → Ala' mutant model peptides studied herein are: (iv) [R22A]-Insulin B-chain; (v) [R17A & R18A]-Glucagon; (vi) [R22A & R24A]-Melittin.

As already mentioned above, in order to explore the factor(s) that limit the applicability of CID for MD approach, we decided to include two smaller peptides as well in this study: (i) Angiotensin II (8 a.a.r., containing 1 Arg) and (ii) Bradykinin (9 a.a.r. having 2 Args), so that the results obtained from longer peptides can be compared with the results of smaller peptides. In other words, we wish to examine, as to, whether Arg has a greater impact on ESI CID MS/MS characteristics of smaller peptides or on the ESI CID MS/MS characteristics of longer peptides?

Thus, we want to understand, as to, how the two factors: (i) peptide's length and (ii) presence of Arg residues in the sequence, influence the peptide's ESI CID MS/MS characteristics. All the ESI CID MS/MS experiments herein have been carried out on a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer. It needs to be noted that there are not many MD proteomic investigations, which have been done using Q-TOF mass spectrometry. So, the role



Scheme 1. Protonation sites on a polypeptide (a) without Arg residue and (b) with Arg residue. The backbone fragment ions: b- and y- ions that can be anticipated upon CID of such polypeptides are also shown.

or utility of Q-TOF mass spectrometry for MD proteomics is still not clear. Hence, it is believed that the results from this study may be helpful to know, the extent of applicability or usefulness of Q-TOF mass spectrometry for MD proteomics.

2. Materials & methods

2.1. Materials

Model peptides such as Angiotensin II (Human, A9525), Bradykinin (Human, B3259), Glucagon (Human, synthetic; G2044), Melittin (Honey bee, M2272), Insulin (Bovine, I5500) were purchased from Merck (Sigma-Aldrich, Bengaluru, Karnataka, India). The reagents, 1,2-Cyclohexanedione (C101400), Phenylglyoxal (142433), Dithiothreitol (DTT; D0632), Iodoacetamide (IAM; I6125) and Formic Acid (FA; 56302), were also bought from Merck (Sigma-Aldrich, Bengaluru, Karnataka, India). The LC-MS grade acetonitrile (ACN; 21301) was bought from s d fine-chem Limited (Bengaluru, Karnataka, India). Boric acid (80266) and Borax (19486) were purchased from Sisco Research Laboratories (Mumbai, Maharashtra, India). Synthetic mutant peptides (custom synthesized) of Insulin B-chain, Glucagon and Melittin were bought from Genscript, Piscataway, NJ 08854, USA and synthetic mutant of Angiotensin II was procured from USV Ltd., Mumbai, India. Water was from Millipore water purification system that is available in-house.

2.2. Methods

2.2.1. Arginine modification of peptides by 1,2-dicarbonyl compounds

Arg modification of the model peptides was done by allowing each model peptide to react with PG or CHD, at equimolar concentration (~10–20 nmol). This Arg modification was carried out directly on Melittin, Glucagon, Angiotensin II and Bradykinin standards; whereas in the case of Insulin, only its B-chain was subjected to modification, since B-chain possesses only one Arg in its sequence. The Insulin B-chain was obtained by carbamidomethylation reaction using DTT and IAM, which was observed in the analytical LC-ESI-MS runs by optimizing the LC gradient (see LC-ESI-MS/MS section). Every Arg modification reaction was monitored at the end of 5 hrs and after ~ 16 hrs, using LC-ESI-MS/MS. All these reactions were done at room temperature (RT: ~ 25–27 °C) using sodium borate (SB) medium at pH 8.4. The data discussed here are from the products obtained over the period of ~ 16 hrs.

2.2.2. Mutant peptides

Synthetic mutant analogs of four model peptides were chosen for this investigation, wherein the Args in these peptides were replaced with alanine (Arg → Ala mutation): (1) Arg at 22nd position of Insulin B-chain replaced with Ala, [R22A]-Insulin B-chain; (2) Arg residues at positions 17 and 18 of Glucagon standard replaced with Ala residues, [R17A & R18A]-Glucagon (double mutant); (3) Arg residues at positions 22 and 24 of Melittin standard substituted with Ala residues, [R22A & R24A]-Melittin (Double Mutant); (4) Arg at 2nd position replaced with Ala in Angiotensin II, [R2A]-Angiotensin II. Every sample (~10–20 nmol) was prepared by dissolving every peptide in SB medium (pH 8.4) and the LC-ESI-MS/MS data were acquired separately for each peptide.

2.2.3. LC-ESI-MS/MS of Arg modified peptides and mutant peptides

Liquid chromatography (reverse phase) – electrospray ionization – tandem mass spectrometry experiments were recorded on 6540 & 6545 Ultra High Definition Accurate-Mass Q-TOF LC/MS attached to 1290 Infinity LC (Agilent Technologies), which has a hexapole collision cell. Agilent Jet Stream (AJS) ESI mode was used and the source parameters were: Gas (N_2) Temperature 300 °C; Gas flow 8 L/min; Nebulizer Pressure 35 psi; Sheath Gas (N_2) Temperature 350 °C; Sheath Gas Flow 11 L/min; VCap +3.5 kV; Nozzle Voltage 1000 V and Fragmentor 175 V. The *m/z* range of detection was set to 300–1700. LC was carried out on Aeris Peptide 2.6 μm XB C-18 (2.1 mm × 100 mm; Phenomenex) column using H_2O (Solvent A) and ACN (Solvent B), each containing 0.1% FA. A linear gradient, 5%–60% solvent B over 15 min was followed at a flow rate, 0.1 ml/min. For every run, ~ 2–10 nmol of peptide was injected on-column. In order to separate and detect the carbamidomethylated B-chain of Insulin, the LC gradient was optimized to 10%–40% of solvent B on the same column. The MS/MS data (Auto MS/MS) on the mutant peptides were acquired following collision induced dissociation (CID), by applying different collision energies (CEs): 25 eV, 35 eV and Ramped, using nitrogen (N_2) as collision gas. For charge state, z = +2, Ramped CE was applied by setting the slope to 3.1 with an offset = 1 and Ramped CE for charge state, z ≥ +3 was applied by setting slope = 3.6 with an offset = - 4.8. CID MS/MS data on the Arg-modified peptides were recorded by applying 'Ramped CE' only. A few CID MS/MS experiments on the standards and mutants were recorded by changing the slope of the Ramped CE. In all LC-MS/MS runs, eight precursor ions that have high intensities at every retention time were allowed to be selected for CID. All the data were processed and analyzed in Agilent MassHunter Workstation Software, Qualitative Analysis Version B.07.00.

3. Results

In our previous study, optimization, specificity and stoichiometry of Arg modification reactions performed on the amino acid, L-Arginine and two model peptides using both the reagents, PG and CHD, were discussed. Moreover, we demonstrated the importance of various reaction mediums in influencing the formation of different types of Arg-modified (Arg-Mod) products [13]. We now want to turn our attention to investigate the effect of Arg on the CID MS/MS spectral features of certain model peptides, particularly of lengths in the range 25– 30 a.a.r., for which the observations from our previous study proved useful as background knowledge, especially for the sake of performing Arg modification reactions on the chosen model peptides. In accordance with the objectives as already delineated in the earlier section, this study is designed in the following manner: (1) Comparison of CID MS/MS characteristics of these model peptide standards versus (vs.) Arg-Mod model peptides, where Arg modification is done by using both PG and CHD; (2) Comparison of CID MS/MS features of these model peptide standards vs. Arg → Ala mutant peptide models. And, the model peptides chosen for this study are: Insulin B-chain (30 a.a.r. having 1 Arg), Glucagon (29 a.a.r. containing 2 Args) and Melittin (26 a.a.r. possessing 2 Args), whose lengths are in the range 25–30 a.a.r. (see Table 1).

From the mass shifts observed in the mass spectral data of Arg-Mod peptides, we found that there are two different types of Arg-Mod products: (a) *Type I* Arg-Mod product: Addition & No Condensation (ΔM = + 112.05 (CHD); ΔM = + 134.04 (PG)) and (b) *Type II* Arg-Mod product: Addition & Condensation (ΔM = + 94.05 (CHD); ΔM = + 116.05 (PG)). We also noted that each Arg residue in the peptide uptakes one molecule of CHD or PG in their respective modification reactions. The MS/MS experiments were conducted on both *Type I* as well as *Type II* Arg-Mod peptide products. Further, MS/MS spectral features observed from Arg-Mod peptides are compared with that of Arg → Ala mutant peptides.

Table 1
List of model peptides used in this study.

S.No	Peptides	Sequence	Mol.Mass (Monoisotopic)
1	Insulin B-chain[#,] ◆	FVNQHLC[#]GSHLVEALYLVC[#]GE**R**GFFYTPKA	3511.71637
2	Glucagon[a,b]	HSQGTFTSDYSKYLDS**RR**AQDFVQWLMNT	3480.61517
3	Melittin[c]	GIGAVLKVLTTGLPALISWIK**R**K**R**QQ - NH_2	2844.75357
4	Arg22Ala - Insulin B-chain[b] ([R22A] - Insulin B-chain)	FVNQHLCGSHLVEALYLVCGE**A**GFFYTPKA	3312.60947
5	Arg17Ala & Arg18Ala - Glucagon[b] ([R17A & R18A] - Glucagon)	HSQGTFTSDYSKYLDS**AA**AQDFVQWLMNT	3310.48717
6	Arg22Ala & Arg24Ala - Melittin[b] ([R22A & R24A] - Melittin)	GIGAVLKVLTTGLPALISWIK**A**K**A**QQ - NH_2	2674.62567
7	Angiotensin II[a,b]	D**R**VYIHPF	1045.53397
8	Arg2Ala - Angiotensin II[b] ([R2A]-Angiotensin II)	D**A**VYIHPF	960.46997
9	Bradykinin[a,b]	**R**PPGFSPF**R**	1059.56087

[#] Carbamidomethyl; ◆ Bovine.
[a] Human.
[b] Synthetic peptide.
[c] Honey bee.

3.1. Model peptides of lengths in the range, 25 a.a.r. - 30 a.a.r.

3.1.1. Carbamidomethyl Insulin B-chain (FVNQHLC[#]GSHLVEALYLVC[#]GERGFFYTPKA)

3.1.1.1. MS/MS of Carbamidomethyl Insulin B-chain (Insulin B-chain[#]) vs. Arg- Mod- Insulin B-chain[#]. The B-chain of Insulin was obtained in carbamidomethyl form from intact insulin (bovine), whereby the A-chain[#] and B-chain[#] (#: carbamidomethyl A and B chains) were separated by reverse phase LC upon following a linear gradient of 10%–40% solvent B (ACN) for 40 min (Materials and Methods). Following our earlier study, Arg modification on Insulin B-chain[#] was known by LC-MS analysis, which showed only one molecule of CHD or PG adding onto the sidechain guanidine moiety of arginine in this peptide (Fig. 1) [13]. Prior to analysis of MS/MS spectra of Arg-Mod products of Insulin B-Chain[#], firstly, it is necessary to interpret the MS/MS data of only the Insulin B-chain[#], which will be the reference for comparing the MS/MS data of Arg-Mod products of Insulin B-chain[#] and also for the MS/MS data of the Arg → Ala mutant peptide, wherein Arg is replaced by Ala at the 22[nd] position of it's sequence.

3.1.1.1.1. MS/MS spectrum of Insulin B-chain[#] only (No Arg modification). In this case, the fifth charge state ($z = +5$) precursor ion m/z 703.35325, showed better yield of fragment ions than the other observed charge states: $z = +2$, $z = +3$, $z = +4$ and $z = +6$. The fragment ions detected in the MS/MS spectrum of $z = +5$ state were: b_2, b_{10} - b_{18}, y_2 - y_5, y_{11} - y_{18} (Fig. 2a).

In order to estimate sequence coverage, the backbone fragment ions detected from the CID of all the precursor ion charge states (viz., +3, +4, +5 and + 6) were combined. In doing so, we found that the middle region of Insulin B-chain[#] has a higher susceptibility to undergo better fragmentation (see Scheme 2) than the extent of fragmentation happening near it's N- and C- termini.

3.1.1.2. MS/MS spectrum of Arg-Mod - Insulin B-chain[#]. In the mass spectrum of CHD-Mod - Insulin B-chain[#], the peak intensities

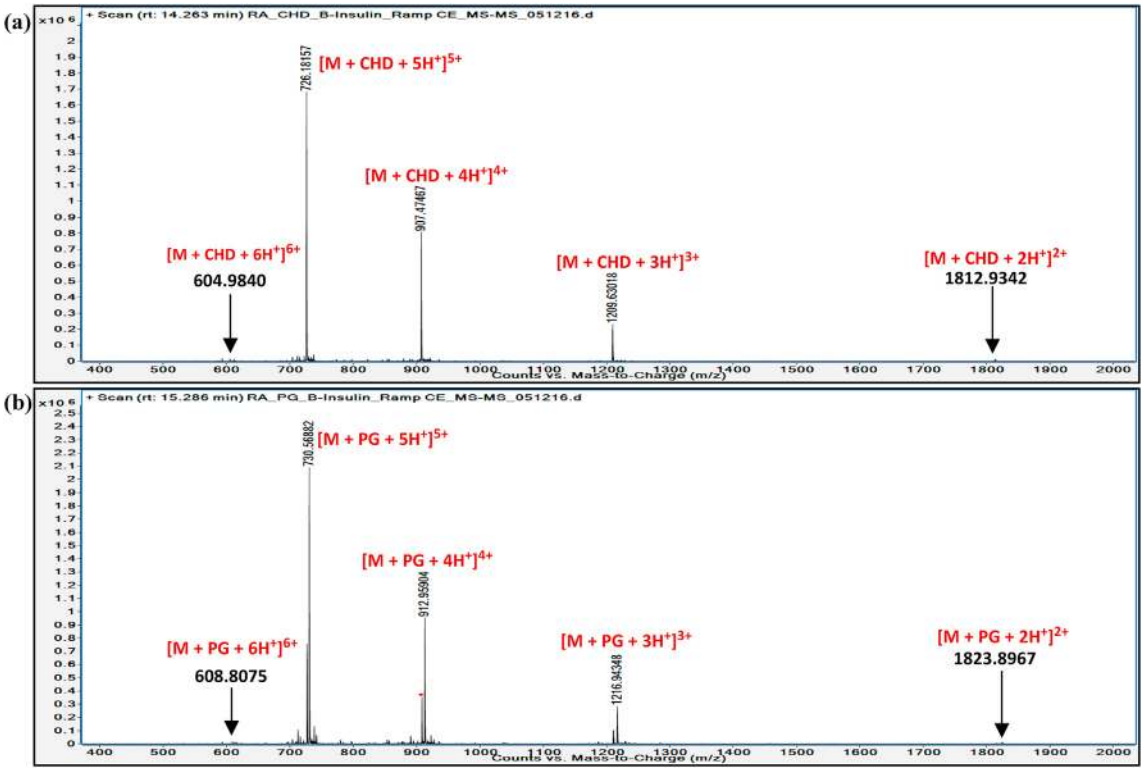


Fig. 1. LC-ESI mass spectrum of (a) CHD-Mod Insulin B-chain[#]; (b) PG-Mod Insulin B-chain[#].

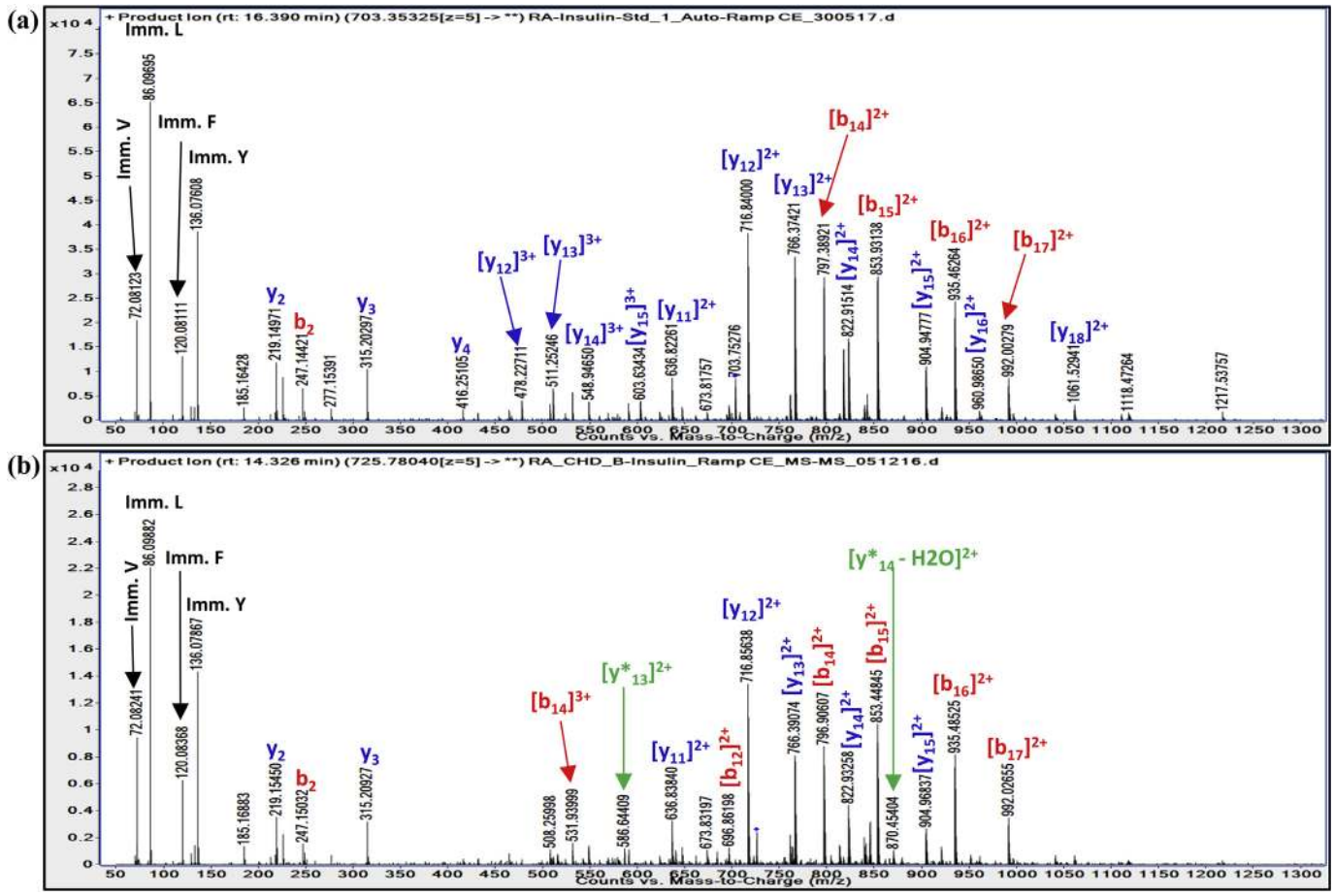
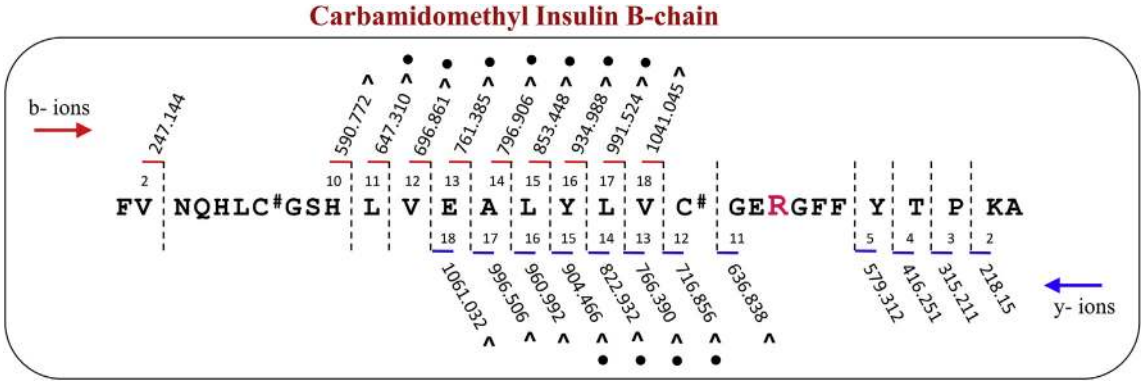


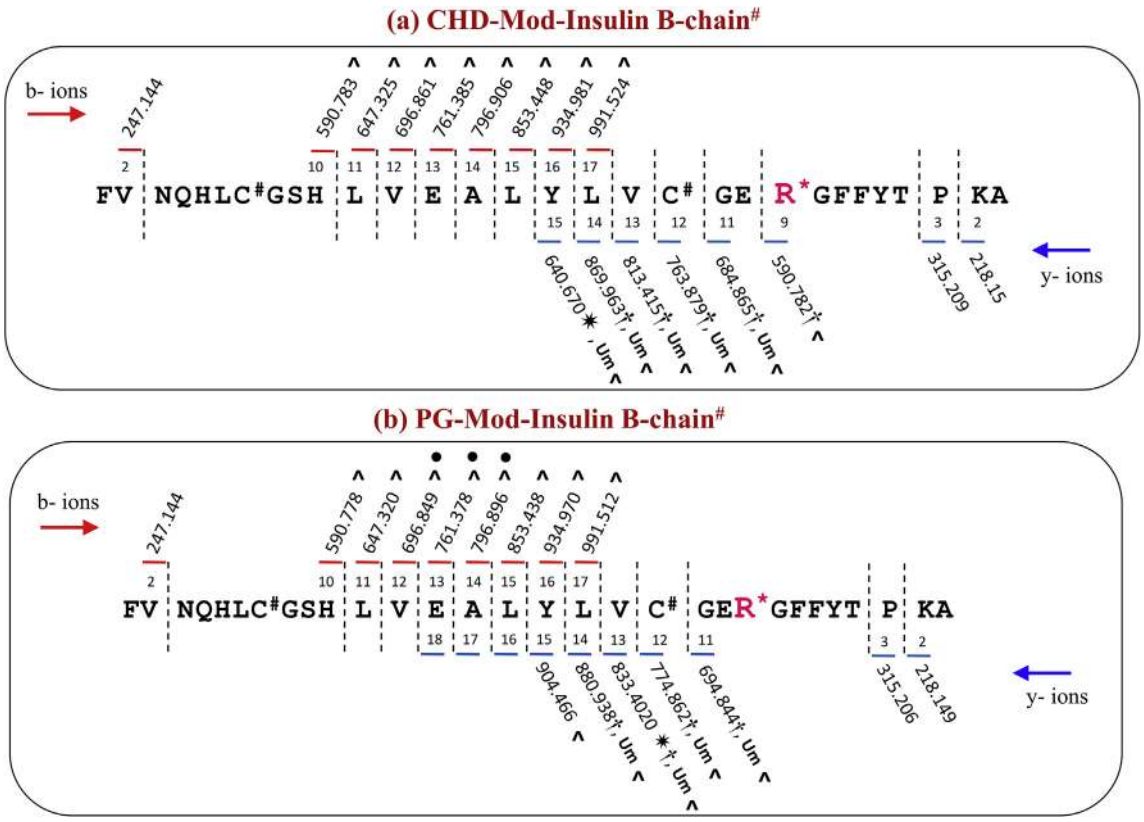
Fig. 2. LC-ESI MS/MS spectrum of (a) Insulin B-chain#, precursor ion $z = +5$; m/z 703.35325; (b) CHD-Mod Insulin B-Insulin#, precursor ion $z = +5$; m/z 725.7804 (#: Carbamidomethyl), acquired by applying Ramped CE.



Scheme 2. Fragmentation pattern of Insulin B-chain# standard (#:Carbamidomethyl),as compiled from the ESI CID MS/MS spectra of all the observed precursor ion charge states; Fragment Ion Intensities > 1000 observed in the MS/MS spectra recorded by applying Ramped CE, were considered to plot this diagram; $\hat{}z = +2$ and $\bullet z = +3$.

of different charge states of *Type I* Arg-Mod - peptide are higher than the respective charge states of *Type II* Arg-Mod - peptide, whereby the modification reaction of this peptide was performed for ~ 16 hrs incubation at room temperature. Therefore, only *Type I* CHD-Mod - peptide (uncondensed product) precursor ions underwent CID MS/MS by means of Ramped CE (Materials and Methods). The yield of fragment ions that resulted from the precursor $z = +5$, m/z 725.780 was found to be better than those obtained from the CID of other charge states: $z = +3$ & $+4$. The fragment ions carrying CHD-Mod moiety as well as fragment ions not having the CHD-Mod group were observed in the MS/MS spectrum of precursor ion $z = +5$: b_2, b_{10} - b_{17}, y_2, y_3, y_{11} - y_{15} are the fragment ions that do not contain the CHD-Mod group, while the fragment ions y^*_9, $y^*_{11} - y^*_{15}$, are the CHD-Mod y-ions that have been detected in their neutral loss form only, viz., [y* - H_2O] (Fig. 2b & Scheme 3a). Interestingly, the CHD-Mod-y_{15} was detected in both the forms, viz., y^*_{15} as well as $[y^*_{15} - H_2O]$.

In the case of PG-Mod Insulin B-chain#, the ionic intensities of



Scheme 3. Fragmentation patterns of (a) CHD-Mod Insulin B-chain#; (b) PG-Mod Insulin B-chain#, prepared by taking different types of b- and y- ions observed in the MS/MS spectra of all the precursor ion charge states (# Carbamidomethyl; Ramped CE; ^ z = +2; · z = +3 ; ✶ Arg-Mod b* & y* ions; † Arg-Mod b* - H_2O & y* - H_2O ions; Um: Unmodified b- & y- ions); Note: $[y^*_{15}]^{3+}$ = 640.670.

both *Type I* and *Type II* Arg-Mod - products were observed to be equal. The MS/MS data of these PG-Mod products were recorded by Ramped CE and we noted that MS/MS of precursor ion z = +5 resulted in better yield of fragment ions than the other precursor ion charge states (Supplementary Fig. S1). In this regard, MS/MS of Type I PG-Mod-product precursor ion, z = +5; *m/z* 730.169, yielded PG-Mod y-ions from y^*_{11} – y^*_{14}, wherein these modified y ions have been detected in their neutral loss form [y* - H_2O] only. b- and y- ions having no Arg-Mod site too were observed in this spectrum. Further, in the same MS/MS spectrum, one Arg-Mod fragment ion: y^*_{13} (*m/z* 833.402) was detected in both y* and [y* - H_2O] forms. MS/MS of *Type II* PG-Mod-product (precursor ion, z = +5; *m/z* 726.568) yielded y^*_{11} - y^*_{14}, y^*_{17}, y^*_{18}, $y^*_{28}$and y^*_{29}, where all these fragment ions were in their neutral loss form, viz., [y* - H_2O]. Additionally, b- and y- ions having no Arg-Mod also were observed in this spectrum.

Intriguingly, b-ions that lacked Arg modifying group were detected, in both the MS/MS of *Type I* and *Type II* Arg-Mod - products (i.e., products of both CHD as well as PG; Table 2). Another aspect is that the intensities of the native b- and y- ions, which lack any type of Arg-modification were noted to be higher than the intensities of Arg-Mod y-ions. This means that the loss of the modifying group (PG or CHD) from the sidechain of Arg is a more preferred dissociative pathway than the peptide backbone fragmentation pathways that give rise to the b- and y- ions. Furthermore, the fragmentation patterns of CHD or PG modified peptides were very similar to that of the Insulin B-chain# (compare Scheme 3 with Scheme 2).

3.1.1.2. MS/MS of Insulin B-chain# vs. [R22A]-Insulin B-chain (Mutant). In order to have a better understanding about the role of Arg modification on the MS/MS characteristics, a synthetic peptide [R22A]-Insulin B-chain also was investigated, in which Arg at 22nd position (R22) in the sequence of the Insulin B-chain standard is replaced with alanine (Ala, A). The ESI charge states distribution observed for the Insulin B-chain# were, z = +2, +3, +4, +5 & +6, while for the R22A mutant peptide, z = +2, +3, +4 & +5 were detected (Supplementary Fig. S2). The peak intensity for the charge state, z = +2 of the mutant peptide was far better than the intensity of the same charge state of the standard Insulin B-chain# (Supplementary Fig. S2). Further, no peak corresponding to z = +6 was detected in the ESI mass spectrum of mutant peptide, whereas Insulin B-chain# showed a peak for z = +6 with lower intensity. These differences in the charge states distribution can be attributed to the replacement of one Arg in the sequence of the peptide standard with Ala. Fig. 3 shows CID MS/MS spectrum of z = +3 of the mutant peptide, which gave rise to a better yield of fragment ions than observed from the CID MS/MS of other higher precursor ion charge states of this mutant peptide. Whereas, in the case of Insulin B-chain#, z = +5 precursor ion resulted in better fragmentation, as discussed above (*vide supra*). No good fragmentation was noticeable from the CID of precursor ion z = +2 of both mutant and standard peptides.

Overall, by combining the details of the fragment ions observed from the MS/MS spectra of precursor charge states, z = +3, +4 & +5, [R22A]-Insulin B-chain showed a far better sequence coverage = 68.96% (33.62%) than the carbamidomethyl form = 51.7% (18.96%), when Ramped CE was applied for effecting CID experiments (Scheme 4 & Table 2). Thus, there is an improvement in the fragmentation efficiency by about 17.26% (14.66%), upon substituting one Arg with an Ala. The values of sequence coverage mentioned in the parentheses have been calculated using

Table 2
Number of observed b- and y-ions and it's sequence coverage[a] in the CID MS/MS spectral data of standard, Arg-Mod and Arg → Ala mutant peptides.

Peptides	Insulin B-chain[#] (30 a.a.r.)			Glucagon (29 a.a.r.)					Melittin (26 a.a.r.)				
Daughter ions	***b***	***y***	**Seq. Cov. (%)**	***b***		***y***		**Seq. Cov. (%)**	***b***		***y***		**Seq. Cov. (%)**
Standard	10	12	51.7	11		4		39.2	14		23		92
Arg → Ala Mutant	20	19	68.96	21		11		75	25		23		96
Daughter ions	***b****	***y****		***b****		***y****			***b****		***y****		
No.of Arg.Mod	**1Arg.**	**1Arg.**		**1Arg.**	**2Args.**	**1Arg.**	**2Args.**		**1Arg.**	**2Args.**	**1Arg.**	**2Args.**	
Unmod	9	8	48.27	18	15	13	12	75 & 60.7	15	14	16	17	69.56 & 68
CHD − Mod	0	6		8	7	4	4		0	0	0	0	
Unmod	9	7	44.8	10	9	5	3	42.1 & 32.1	7	Nil	12	Nil	56
PG − Mod	0	4		4	4	0	0		0	Nil	1	Nil	

[#] Carbamidomethyl; **b***: Arg-Mod b ions;**y***: Arg-Mod y ions; Nil: Precursor ions not selected for CID.
[a] Note: (1) Sequence Coverage: '**Seq. Cov. (%)**' values shown in this Table are calculated using the formula proposed below: **Seq. Cov. (%)** = (No.of backbone peptide bonds that were observed to undergo fragmentation / Total No.of backbone peptide bonds in the polypeptide) × 100. (2) In the cases of Arg-Mod Glucagon and Arg-Mod Melittin, two sequence coverage values have been mentioned, which correspond to the One-Arg Mod.Peptide and Two-Args Mod.Peptide, respectively.

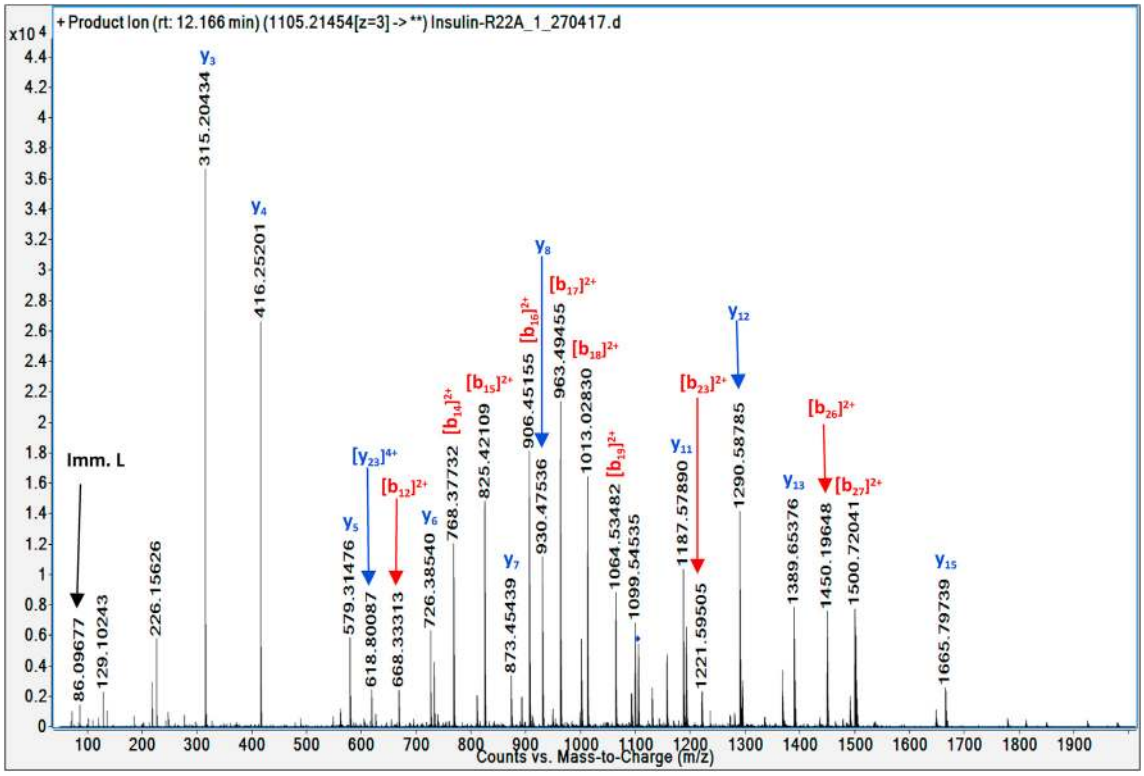


Fig. 3. LC-ESI MS/MS spectrum of [R22A]-Insulin B-chain, precursor ion $z = +3$; m/z 1105.21454; acquired by applying Ramped CE.

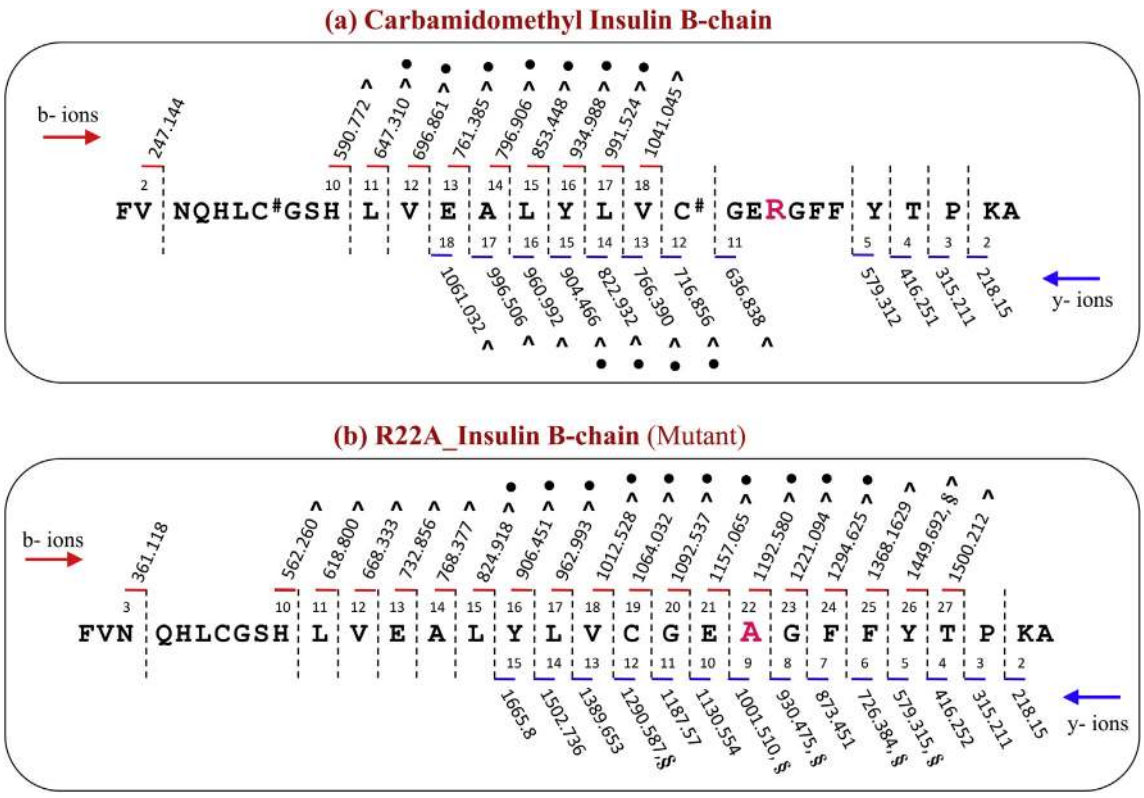
a different formula, as shown in Supplementary Table S1.

3.1.2. *Glucagon* (HSQGTFTSDYSKYLDSRRAQDFVQWLMNT)

3.1.2.1. MS/MS of Glucagon Standard vs. Arg-Mod - Glucagon

3.1.2.1.1. MS/MS spectrum of Glucagon Std. (No Arg modification). Glucagon is another model peptide (hormone) consisting of 29 a.a.r., which has two arginines positioned very near to the middle portion of it's sequence, i.e., R17 and R18. On performing CID MS/MS experiments on the Glucagon Standard (Glucagon Std.) using Ramped CE, better backbone fragmentation was observed from the charge state z = +4 than the other observed charge states, z = +2,+3 and +5. Indeed, the charge state z = +4 showed a remarkable fragmentation efficiency in that most of the peptide backbone units underwent dissociation giving rise to 22 b-ions: b_1-b_7, b_9-b_{11}, b_{15}, b_{18}, b_{20}-b_{28} and 18 y-ions: y_1-y_6, y_{14}, y_{16}-y_{26}, except that no fragmentation was observed at three peptide units: S16 - R17, R17 - R18 and A19 - Q20, which works out to ~ 50% of backbone fragment ions getting detected upon CID in Q-TOF (Supplementary Fig. S3). Thus, this MS/MS spectrum was considered as a reference to compare with the MS/MS spectral data of CHD-Mod- and PG-Mod- Glucagon by applying the same Ramped CE.

3.1.2.1.2. MS/MS spectrum of Arg-Mod - Glucagon. After the chemical modification reactions, the mass shifts observed in the mass spectral data, confirmed that both *Type I* and *Type II* Arg-Mod products were formed with both CHD as well as PG. Additionally, the peak shifts in the mass spectra also indicated the formation of One-Arg-Mod - Glucagon and Two-Args-Mod - Glucagon: (a) only R17 modified or only R18 modified (R*17 or R*18; Scheme 5) and (b) both R17 & R18 modified (R*17 & R*18). Moreover, no significant changes were observable in the ESI charge states distribution due to modification of both the arginines (viz., R*17 & R*18) of Glucagon by CHD or PG. In other words, the ESI charge states distributions of the Arg-Mod products were almost similar to that of Glucagon Std., even though two molecules of CHD or PG modified



Scheme 4. Comparison of fragmentation patterns of (*a*) Insulin B-chain# (#: Carbamidomethyl) and (b) [R22A]-Insulin B-chain, as compiled from the ESI CID MS/MS spectra of all the observed precursor ion charge states; Fragment Ion Intensities > 1000, observed in the MS/MS spectra recorded by applying Ramped CE, were considered to plot this diagram;^$z = +2$; •$z = +3$ and §:b-H_2O/y-H_2O).

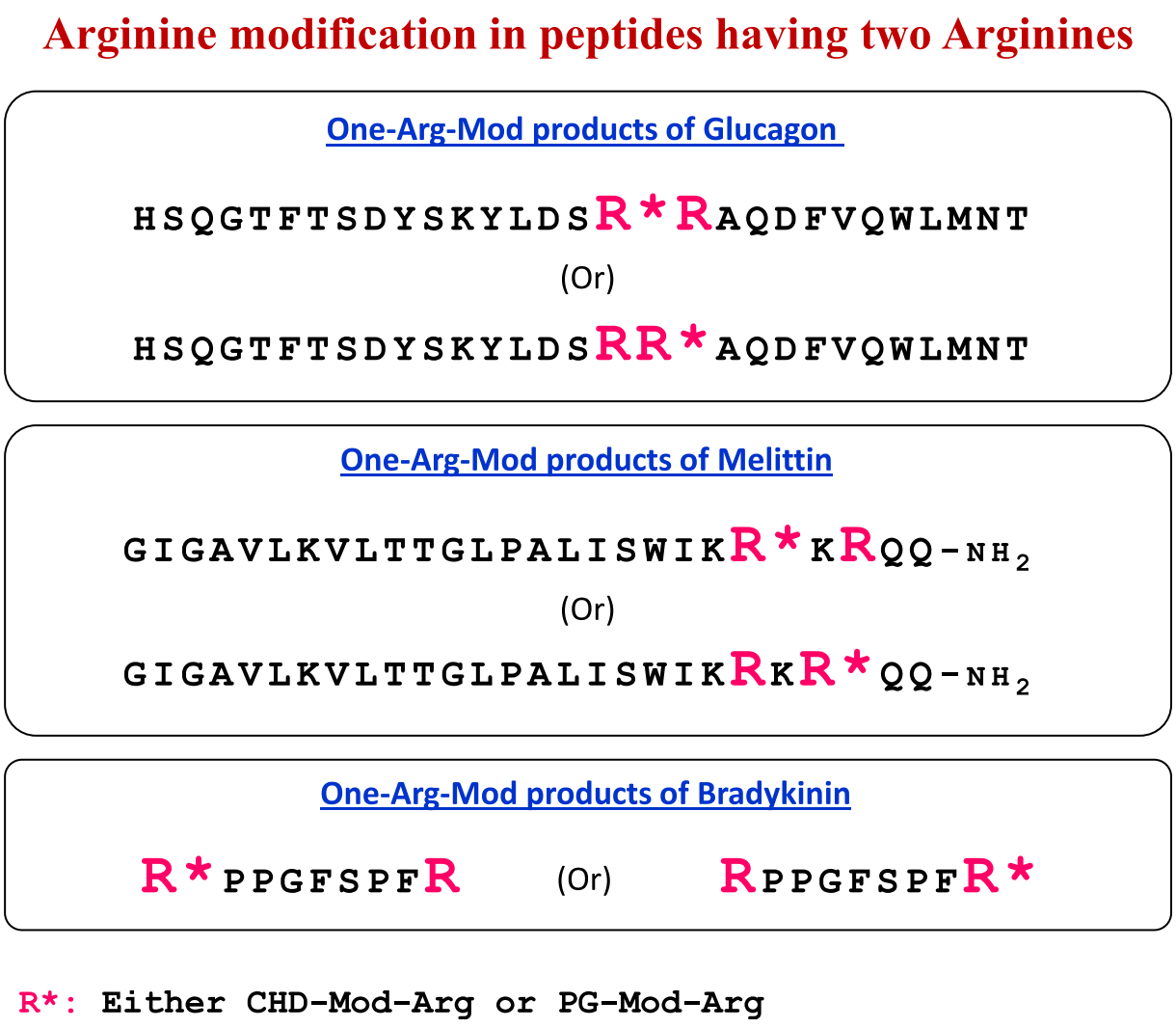
the Glucagon (Supplementary Fig. S4). Further, the peak intensities of *Type II* Arg-Mod products were found to be significantly lower than those of *Type I* Arg-Mod products, which were observed in both CHD-Mod- and PG-Mod products. Therefore, precursor ions of only *Type I* products of Arg-Mod - Glucagon were subjected to MS/MS experiments by applying Ramped CE (Materials and Methods). The CID MS/MS spectrum of *Type I* products of both One-Arg-Mod-(R*17 or R*18) and Two-Args-Mod- (R*17 & R*18) Glucagon revealed two key fragmentation processes: (1) neutral loss of H_2O from their respective modified precursor ion *m/z* and (2) neutral loss of the Arg-Mod group (i.e., CHD or PG), giving rise to good peak intensities corresponding to the standard Glucagon.

The MS/MS spectrum of z = +4 (precursor ion *m/z* 927.1884) of Two-CHD-Mod (R*17 & R*18) Glucagon, gave rise to better fragment ions' yield than the other precursor ion charge states (see Supplementary Fig. S5). The yield of b- and y- ions obtained from the CID MS/MS spectra of all the detected charge states of both One-CHD-Mod-(R*17 or R*18)-Glucagon and Two-CHD-Mod-(R*17 & R*18)-Glucagon, showed similar fragmentation patterns (Supplementary Scheme S1). The b- and y- ions having the CHD-Mod-group were noted to be predominantly in neutral loss forms, i.e., [b*- H_2O], [y* - H_2O]; where b* and y* are CHD-Mod- b- and CHD-Mod- y-ions, respectively (Table 2). The observed b*, [b* - H_2O], y* and [y* - H_2O] ions that were interpreted from the MS/MS spectra of One-CHD-Mod-(R*17 or R*18)-Glucagon and Two-CHD-Mod-(R*17 & R*18)-Glucagon have been summarized in Table S2 (Supplementary Material).

It is important to note that the number of observed CHD-Mod-fragment ions (b*, [b*- H_2O], y* and [y*-H_2O] ions) were less than the number of b- and y-ions that were devoid of the CHD-Mod-group (Table 2). This observation prompted us to infer that the loss of CHD-Mod-group is a more facile dissociative process during CID in the Q-TOF mass spectrometer used in this study.

In the case of PG-Mod- Glucagon, the fragmentation efficiency of *Type I* PG-Mod precursor ion was better than the *Type II* PG-Mod precursor ion. In the case of *Type I* PG-Mod products, we did not see drastic differences between the CID MS/MS fragmentation of One-PG-Mod- (R*17 or R*18) Glucagon and Two-PG-Mod- (R*17 & R*18) Glucagon (Supplementary Fig. S6 and Supplementary Scheme S2). As already mentioned above, the major dissociative pathway during the course of CID of *Type I* PG-Mod- Glucagon was the neutral loss of PG from their respective precursor ions (z = +3, +4 and + 5). And this was evident from the observation of more number of unmodified b-ions than the PG-Mod-b- ions in the MS/MS spectrum of PG-Mod- Glucagon; whereby the intensities of all the PG-Mod- b ions were less than the peak intensities of unmodified b- and y- ions (Supplementary Fig. S6). Unmodified b-ions detected were: $\mathbf{b_2}$, $\mathbf{b_5}$ - $\mathbf{b_7}$, $\mathbf{b_{15}}$, $\mathbf{b_{22}}$ - $\mathbf{b_{28}}$ and $\mathbf{y_1}$ **-** $\mathbf{y_4}$, $\mathbf{y_{13}}$. Arg-Mod b* ions were: $\mathbf{b^*_{22}}$, $\mathbf{b^*_{24}}$ - $\mathbf{b^*_{26}}$, which were in their neutral loss form [b* - H_2O] (Supplementary Scheme S2). With respect to y-ions, no PG-Mod- y ions were observed; only $\mathbf{y_2}$ and $\mathbf{y_3}$ ions were detected (Table 2).

Altogether, by comparing the ESI CID MS/MS spectral data of Arg-Mod - Glucagon products (both CHD and PG) with that of the Glucagon Std., it was realized that Arg modification did not have a significant influence in altering the fragmentation pattern of the Glucagon Std. But, relatively a better sequence coverage was found in the case of CHD-Mod - Glucagon than the sequence coverage estimated for the PG-Mod - Glucagon.



Scheme 5. Different types of Arg-Mod products that can be anticipated in peptides having two Arginines.

3.1.2.2. MS/MS of Glucagon Std. vs. [R17A & R18A]-Glucagon (Double Mutant). [R17A & R18A]-Glucagon (Double Mutant) is a synthetic peptide, where the two arginines at 17th and 18th positions in the sequence of the Glucagon Std. have been substituted with two alanines. The ESI charge states distribution of the double mutant and the standard peptides were same, showing z = +2, +3, +4 & +5. The peak intensity of z = +5 of the double mutant was however, significantly lower than the intensity of z = +5 of the peptide standard (unmodified). Furthermore, the precursor ion z = +2 of both the standard as well as the mutant peptides, was not found to undergo CID properly, as inferred from the precursor ion intensity in their respective MS/MS spectrum. Consequently, the MS/MS spectra acquired on the precursor ions of charge states z = +3 & +4 for the double mutant were considered for further interpretation. The MS/MS experiments of standard and mutant Glucagon were done using different CEs such as 25 eV, 35 eV, Ramped CE, where two different slopes were applied for Ramped CE. Correspondingly, sequence coverage was also estimated for both the peptides. Thus, for every type of CE (i.e., Fixed CE and Ramped CE), the fragmentation pattern and the sequence coverage was derived for both the peptides. Among various CE types applied (*vide supra*), it was found that at CE = 25 eV, the fragmentation efficiency of the standard peptide was significantly different from that of the double mutant Glucagon, whereby the double mutant showed a better yield of fragment ions than the standard (Supplementary Fig. S7 and Supplementary Scheme S3). But, the fragmentation pattern of the Glucagon Std. was not drastically different from the dissociation pattern of the double mutant Glucagon, when Ramped CE was applied for CID MS/MS experiments (Supplementary Scheme S4). Altogether, irrespective of the CE, better sequence coverage was obtained for the double mutant peptide. Besides the influence of different CEs, the nature of charge state of the precursor ion too impacted the fragmentation pattern and the extent of sequence coverage. In the case of double mutant, the MS/MS of z = +3 precursor ion gave rise to better sequence coverage and for the Glucagon Std., MS/MS of z = +4 precursor ion resulted in better sequence coverage. In Glucagon Std., the fragmentations mainly occurred better near the N- and C- termini, as noted from the 11 fragment ions: b_2, b_3, b_5, b_6, b_9, b_{22} - b_{27}; y_2-y_4; sequence coverage = ~ 39.2% (19.64%) (Table 2 & Supplementary Table S1). As discussed above, mutant Glucagon showed better fragmentation efficiency, which yielded 21 b-ions (b_2, b_5, b_9, b_{10} and b_{12} - b_{28}) and 11 y-ions (y_2 - y_{12}); which corresponded to the sequence coverage = ~ 75% (31.25%) (Table 2 & Supplementary Table S1). Thus, the sequence coverage enhances by approx. 1.5–1.9 times, when the two arginines are replaced by two alanines, when Fixed CE = 25 eV is considered (Supplementary Scheme S3).

3.1.3. *Melittin* (GIGAVLKVLTTGLPALISWIKRKRQQ - NH_2)

3.1.3.1. MS/MS of Melittin Standard vs. Arg-Mod - Melittin

3.1.3.1.1. MS/MS spectrum of Melittin Std. (No Arg modification). Melittin is a C-terminal amidated 26 a.a.r. long peptide, which consists of two arginines at position 22nd and 24th (R22 and R24). The charge state distribution in the ESI mass spectrum of Melittin was: z = +2, +3, +4, +5 and + 6, among which z = +4 and + 5 gave better fragmentation efficiency during CID MS/MS. These MS/MS experiments were attempted in different CE conditions: Fixed CE at 20 eV, 25 eV, 30 eV and Ramped CE by varying the slope. It was found that Fixed CE = 25 eV, gave better fragmentation, yielding 14 b- ions: b_2 - b_{15} and 23 y- ions: y_2 - y_{24} (Supplementary Scheme S5a).

3.1.3.1.2. MS/MS spectrum of Arg-Mod - Melittin. Similar to the case of Glucagon, Melittin also can form different Arg-Mod products due to modification by PG or CHD, since Melittin also contains two arginines. The products are: (1) One-Arg-Mod - Melittin that can be R*22 - Melittin or R*24 - Melittin (see Scheme 5) and (2) Two-Args-Mod -Melittin, which is [R*22 & R*24] - Melittin. Akin to the previously described arginine modified peptides, in the case of Arg-Mod - Melittin also, both *Type I* and *Type II* Arg-Mod products were observed from both One-Arg-Mod and Two-Args-Mod products. Moreover, here also the ionic intensities of *Type I* Arg-Mod products were higher than that of the *Type II* Arg-Mod products. But, in the case of Two-PG-Mod - Melittin (R*22 & R*24), the ionic intensities of *Type II* product was found to be higher than the ionic intensities of *Type I* product. Therefore, precursor ions of only *Type I* Arg-Mod - Melittin products were taken for CID MS/MS experiments, whereas in the case of Two-PG-Mod - Melittin, precursor ions of *Type II* product also was subjected to CID. Further, the charge state distributions of the all the Arg-Mod products were same as that of the Melittin Std., viz., z = +2 - +6 (Supplementary Fig. S8).

With regard to the both One-CHD-Mod- and Two-CHD-Mod - Melittin, precursor ion z = +5 gave better fragmentation than the other precursor ion charge states, under the conditions of Ramped CE (Supplementary Fig. S9a). Inspection of fragment ions detected from all the precursor charge states (viz., z = +3, +4, +5 and + 6) of both One-CHD-Mod- and Two-CHD-Mod- Melittin, it was observed that no b* and no y* were detected at all. Only unmodified b- and y-ions from both One-CHD-Mod - Melittin and Two-CHD-Mod - Melittin were observed, which are: 14 b- ions (b_2 - b_{15}) and 17 y-ions (y_8 - y_{24}) (Supplementary Schemes S6a and S6b). And no significant differences were conspicuous between the CID patterns of Melittin Std. and CHD-Mod - Melittin products.

The CID MS/MS of One-PG-Mod - Melittin too gave rise to only unmodified b- and y- ions (b ions: b_2-b_5, b_8, b_{12}, $b_{13;}$ y ions: y_9-y_{13}, y_{15}-y_{18}, y_{21} - y_{23}) (Supplementary Fig. S9b). However, all types of PG-Mod - Melittin products did not undergo CID very efficiently, in comparison to CID of CHD-Mod - Melittin products and Melittin Std. In other words, CID MS/MS of PG-Mod -Melittin products yielded poorer sequence coverage, when Ramped CE is applied (Supplementary Scheme S6c). So, it may be inferred that modification of sidechain guanido group of Args by PG is hindering the efficiency of backbone peptide fragmentation. Due to limited sample quantity of Arg-Mod products of Melittin, CID experiments by applying Fixed CEs were not done.

3.1.3.2. MS/MS of Melittin Std. vs. [R22A & R24A]-Melittin (Double Mutant). [R22A & R24A]-Melittin is the synthetic mutant analog of Melittin Std., in which both Arg residues at 22nd and 24th positions are replaced by alanines. The ESI charge state distribution of this double mutant Melittin differs from that of the Melittin Std. The standard peptide showed the charge states: z = +2 to +6 (*vide supra*), whereas the double mutant Melittin gave rise to z = +1 to +4. Among all the charge states observed in the ESI-MS of the double mutant Melittin, z = +3 (*m/z* 892.556) underwent CID more efficiently (see Supplementary Fig. S10); whereas z= +4 (*m/z* 712.196) of Melittin Std. showed better fragmentation (data not shown). The precursor ion z = +2 of the mutant did not undergo CID properly. The CID experiments of both the standard and double mutant Melittin were carried out at various CEs such as 20 eV, 25 eV, 30 eV and Ramped CE, from which it was noticed that irrespective of CE, mutant Melittin yielded higher sequence coverage than the Melittin std., whereby better fragmentation efficiency was observed at CE 25 eV (Supplementary Scheme S5).

3.2. Model peptides of lengths in the range 5 a.a.r. - 10 a.a.r.

In order to understand the effect of Arg on the CID MS/MS characteristics (fragmentation pattern and sequence coverage) of the smaller-sized peptides that adopt lower precursor ion charge states (z = +1 and +2), Angiotensin II (8 a.a.r.) and Bradykinin (9 a.a.r.) are chosen for this study. In fact, MS/MS characteristics of these two model peptides have been reported by Leitner & Lindner, but they had utilized a different Arg modifying tag, 2, 3 - butanedione (BD) along with boronic acid [14,15]. So, the molecular structure of the precursor ions of the Arg-Mod - peptides that they subjected for MS/MS experiments was entirely different from the structure of the Arg-Mod - products that we have obtained here. This is because the reagent (BD) and the medium that they had used for Arg modification reactions are different from the reagents (CHD & PG) and the medium (SB) that we have used in this study. Moreover, their MS/MS experiments were carried out in a triple quadrupole or an ion trap mass spectrometer, whereas here, all of our CID MS/MS experiments have been done in a Q-TOF mass spectrometer.

3.2.1. *Angiotensin II* (DRVYIHPF)

3.2.1.1. MS/MS of Angiotensin II Std. vs. Arg-Mod - Angiotensin II. Arg is present near to the N-terminus region of this peptide, which underwent modification in both the PG and CHD modification reactions. As we reported previously, in both the Arg modification reactions, 4 hrs reaction resulted in the formation of predominantly *Type I* Arg-Mod - products; whereas longer reaction periods, e.g., ~ 16 hrs gave rise to the formation of *Type II* Arg-Mod products as well [13]. However, the intensity of *Type I* Arg-Mod – products were higher than the *Type II* Arg-Mod products. Therefore, all of our MS/MS experiments were conducted on only the *Type I* Arg-Mod - Angiotensin II products derived from CHD or PG modification (Supplementary Fig. S11).

There were no drastic alterations to the ESI charge states distribution between the standard peptide and the Arg-Mod - peptides; whereby z = +1, +2 and +3 were the observed charge states in the ESI mass spectra of Arg-Mod - peptides. Among these charge states, precursor ion z = +2 underwent better extent of CID (Ramped CE, see Materials and Methods), in the cases of standard as well as Arg-Mod - Angiotensin II products. The sequence coverage of the CHD-Mod- Angiotensin II was very similar to that of the standard peptide, but the PG-Mod- Angiotensin II did not yield good sequence coverage (Supplementary Scheme S7). Indeed, the CHD-Mod- Angiotensin II (*Type I* CHD-Mod-product, *m/z* 579.7748, z = +2) studied herein shows better sequence coverage than the sequence coverage of BD modified Angiotensin II reported by Leitner and Lindner [14,15].

More number of CHD-Mod-b and y ions (b* & y* ions) were detected than PG-Mod b* and y* ions (see Supplementary Scheme S7). Further, the neutral loss of CHD and PG from the respective Arg-Mod peptide precursor ion was noted even at CE = 15 eV, suggesting a weak covalent binding of CHD or PG with the guanido group, which results in the observation of unmodified b and y ions too.

3.2.1.2. MS/MS of Angiotensin II Std. vs. [R2A]-Angiotensin II. The ESI mass spectrum of [R2A]-Angiotensin II contained peaks corresponding to z = +1 & +2 charge states, whereas the Angiotensin Std. showed z = +1, +2 & +3 charge states upon ESI (*vide supra*). This can be attributed due to the Arg → Ala substitution in it's sequence. The MS/MS spectra were acquired for both the standard and [R2A]-Angiotensin II at CE = 25 eV (Supplementary Fig. S12) and it was found that precursor ion z = +2 of the standard peptide underwent better fragmentation, but in the case of mutant, precursor ion z = +1 showed better fragmentation. However, both [R2A]-Angiotensin II and the standard peptide yielded similar sequence coverage (Supplementary Scheme S7). Furthermore, the sequence coverage of CHD-Mod- Angiotensin II was almost similar to that of [R2A]-Angiotensin II. So, with regard to the sequence coverage of Angiotensin II, the effect caused due to modification of sidechain guanido group of Arg by CHD is perhaps very similar to the effect of Arg → Ala substitution. .

*3.2.2. **Bradykinin** (RPPGFSPFR)*

3.2.2.1. MS/MS of Bradykinin Std. vs. Arg-Mod - Bradykinin. Bradykinin is another popular model peptide, which has Arg at both the N- and C- termini of it's sequence. Therefore, Arg modification by CHD or PG can give rise to products such as One-Arg-Mod (R*1 or R*9) Bradykinin and Two-Args-Mod (R*1 & R*9) Bradykinin (refer Scheme 5). Similar to the earlier discussed peptide models, in this case also the ESI charge state distribution: z = +1, +2 and + 3 of all the Arg-Mod - Bradykinin products (R*1-Bradykinin, R*9- Bradykinin, R*1 & R*9-Bradykinin) did not differ from that of the standard peptide. Further, the precursor ion z = +2 of the standard peptide as well as all the Arg-Mod - Bradykinin products were found to undergo good fragmentation upon CID (Supplementary Figs. S13 and S14). Like the previously described peptide models, in the case of CHD-Mod-Bradykinin, the precursor ions of only *Type I* products corresponding to both One-Arg-Mod (R*1 or R*9) Bradykinin and Two-Args-Mod (R*1 & R*9) Bradykinin showed good fragment ion yield due to CID, although peaks of both *Type I* and *Type II* CHD-Mod bradykinin were observed in the conventional ESI mass spectrum. However, in the case of PG-Mod-Bradykinin, precursor ions of both *Type I* and *Type II* products were selected for CID MS/MS, but the MS/MS spectrum of only *Type I* PG-Mod-Bradykinin was found to be good for further interpretation. Irrespective of the modification by CHD or PG, the MS/MS of Two-Args-Mod (R*1 & R*9) Bradykinin yielded fragment ions that had better intensities than the fragment ion intensities in the MS/MS spectra of One-Arg-Mod- (R*1 or R*9) Bradykinin. Detection of b^*_1 and y^*_1 ions with good intensities in the MS/MS spectra of Two-Args-Mod- (R*1 & R*9) Bradykinin suggested that both the Arg residues, R1 and R9 had been modified by CHD or PG. Overall, unlike the other peptide models investigated herein, both PG-Mod - Bradykinin and CHD-Mod- Bradykinin exhibit similar fragmentation efficiency (Supplementary Scheme S8).

MS/MS studies on Arg-Mod - Bradykinin have indeed been reported in the past, wherein the Arg modification was done by CHD, PG and BD [14–16]. However, the 'molecular structures' of the Arg-Mod - peptide precursor ions obtained in those earlier studies were different from the molecular structures of the Arg-Mod - peptide precursor ions that we have obtained here. Wanigasekara and Chowdhury had performed all the CID MS/MS experiments (ESI-Ion Trap (IT)-TOF and MALDI- IT- TOF) on only *Type II* Arg-Mod - products, which they obtained with PG and CHD. However, here, we got very good yield of *Type I* Arg-Mod - peptide products as noted in our conventional mass spectra, which prompted us to explore their respective CID MS/MS properties. Moreover, we have utilized sodium borate as the reaction medium to perform Arg modification involving PG or CHD, whereas Wanigasekara and Chowdhury had used potassium bicarbonate for reactions involving PG and sodium hydroxide for the reactions with CHD [16]. So, the nature of reaction medium employed for Arg modification reactions seems to influence the formation of different types of Arg-Mod products. Furthermore, the fragment ions' yields obtained from the MS/MS of PG-Mod and CHD-Mod - Bradykinin in this study are far better than the fragment ion yield obtained by Leitner and Lindner, where they had used BD for Arg modification of Bradykinin [14,15].

4. Discussion

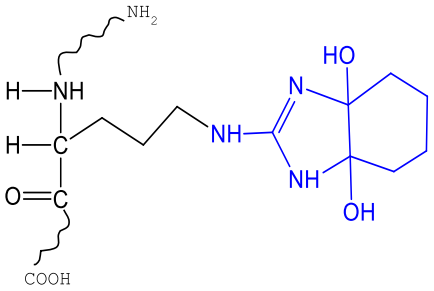
Many biochemical studies have employed 1,2-dicarbonyl compounds such as CHD, PG and BD with the objective to identify active site Arg residues in certain enzymes that are capable of recognizing anionic substrates, e.g., kinase, phosphatase, etc. [17–25]. And, in some investigations CHD and PG have been used to comprehend the role of Arg in influencing the structural or conformational changes of proteins and peptides, e.g., to determine the surface-exposed reactive Arg sites in proteins [16,26,27]. In addition, recently, Chowdhury's group have demonstrated the utility of CHD with azide coupling for selective enrichment of Arg containing peptides from complex mixture of proteins [28]. Further, a few studies have been conducted to explore the effect of Arg modification by BD and malondialdehyde, on ESI CID MS/MS spectral characteristics of peptides [14,15,29]. Herein, we have used CHD and PG for Arg modification, to understand the impact of such Arg modifications on the ESI CID MS/MS behavior of peptides, but our objective is particularly focused on the peptides of lengths in the range 25–30 a.a.r., because we want to obtain ESI charge states in the range: $+3 \leq z \leq +6$ and the peptides of such sizes are capable of adopting these ESI charge states. The key findings of this study are as follows.

Upon modification by CHD or PG, the sidechain guanido group of Arg would resemble an imidazole like structure, due to which there can be alteration in the isoelectric point (pI) of the peptide. Therefore, the 'arginines' in the peptide sequence were considered as 'histidine' residues, viz., the 'Arg' residues were replaced with 'His' residues and then the theoretical pI was calculated for Arg-Mod peptides (see Table 3). Although, there is decrease in the theoretical pI values due to Arg modification (Arg → His), there were no drastic alterations observed in the ESI charge state distributions of all the model peptides studied herein because of Arg modification. This suggests that modification by CHD or PG is not decreasing the basicity of the sidechain guanido group, as noted from the conventional ESI mass spectral data.

With regard to the nature of products obtained by Arg modification, we observed two different types of products: *Type I* and *Type II*, with CHD or PG, whereby all the reactions were conducted in sodium borate buffer (~ 16 hrs, room temperature). The molecular masses of *Type II* products were 18 Da less than the respective *Type I* products, clearly indicative of loss of H_2O (condensation) from *Type I* products. In the ESI mass spectra of all the Arg-Mod peptides, the intensities of *Type I* products were greater than the intensities of *Type II* products. Consequently, the precursor ions of *Type I* Arg-Mod products were mostly chosen for the CID MS/MS data acquisitions. Detecting these two different types of products is perhaps a unique aspect of our study, since most of the earlier published investigations have observed only *Type II* Arg-Mod product [16,21,30].

In the peptides having two arginines in their respective sequence, i.e., glucagon, melittin and bradykinin, we observed different kinds of products: (1) modification of only one arginine, One-Arg-Mod peptide and (2) modification of both the arginines, Two-Args-Mod peptide. In all these Arg-Mod peptides, both *Type I* and *Type II* products were detected.

Table 3
[a]Theoretically calculated isoelectric point (pI) for: (1) Native (No Arg modification); (2) Arg → Ala Mutant; and (3) Arg-Modified Peptides.

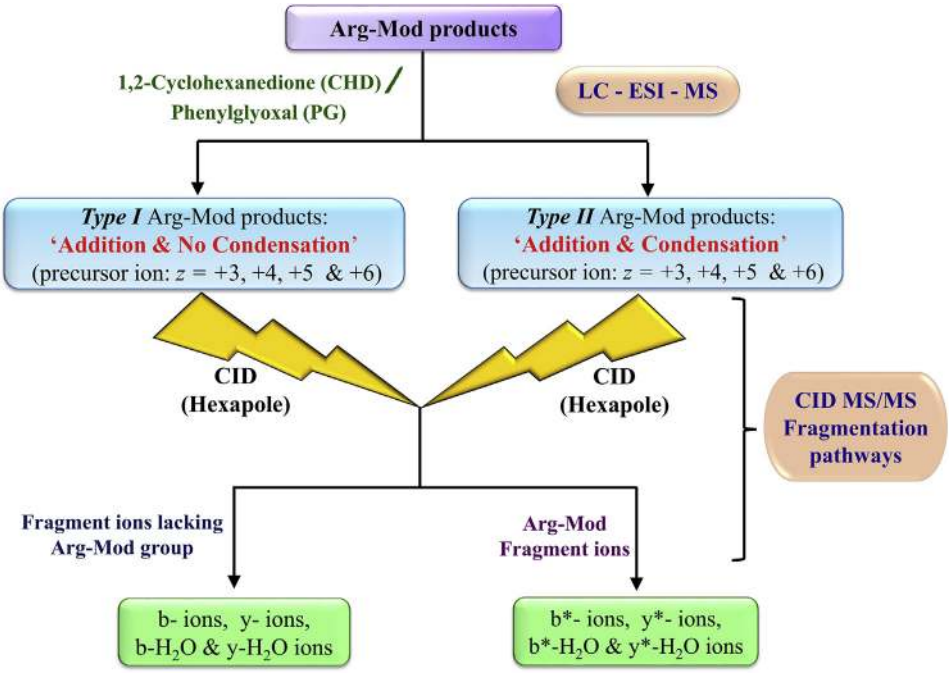


Peptides	Native Arg	Arg → Ala Mutant	Arg Modified → His[b]	
			1Arg → His	2Arg → His
Insulin B-chain[#]	6.9	6	6.3	–
Glucagon	6.75	4.41	5.9	5.71
Melittin	12.02	10.3	11.17	10.3

[a] pI calculation was done using Expasy Protparam (https://web.expasy.org/compute_pi/).
[b] The modified sidechain guanido group of Arg, due to PG or CHD, resembles 'imidazole' like group (see the molecular structure shown above). Hence, to calculate pI values for Arg-Mod peptides, the'arginine' residues were considered as 'histidine' residues; viz., Arg residues were replaced with His residues in Arg-Mod peptides to calculate pI values for Arg-Mod peptides.

With regard to the MS/MS of Arg-Mod peptides, removal of the modifying group from the sidechain guanidine moiety (in the form of neutral loss) seemed to be the predominant fragmentation process taking place during CID (Scheme 6). This suggests that the strength of the covalent binding of CHD or PG to the guanido group is perhaps not strong, as understood from the CID MS/MS data acquired from the Q-TOF mass spectrometers used in this study that houses hexapole collision cell. Nevertheless, we did find b* and y* ions that possessed the Arg-Mod group (CHD or PG), but these b* and y* ions were mostly detected in the form of b* - H_2O and y* - H_2O ions (Scheme 3; Supplementary Schemes S1, S2, S7 & S8). Further, the intensities of such [b*- H_2O] and [y* - H_2O] ions were less than the intensities of the b and y ions that did not carry the Arg-Mod group. Wanigasekara and Chowdhury also reported about the detection of [b* - H_2O] and [y* - H_2O] ions (by PG and CHD), however they did CID MS/MS experiments on the *Type II* Arg-Mod precursor ions only of smaller sized model and tryptic peptides of lengths < 20 a.a.r., using an ion trap - TOF (Shimadzu Corporation) [16]. Moreover, their study was not intended to analyze the impact of Arg modification on the extent of CID MS/MS or sequence coverage [16]. Further, the ESI CID MS/MS studies reported by Leitner and Lindner indicated that Arg modification by BD did not lead to any improvement in the sequence coverage of certain smaller-sized peptides [14,15]; while this study shows that PG or CHD modified guanido group is not significantly aiding in improving the CID of the 'medium-sized' peptides.

Studies conducted on Arg → Ala mutant model peptides revealed that the ESI charge states distributions of these peptides were markedly different from that of the standard peptides (Supplementary Fig. S15), encompassing the range: $+2 \leq z \leq +6$. However, the intensities of precursor ion charge states: $z = +5$ and + 6 of the mutant peptides were not sufficiently higher to be chosen for CID MS/MS experiments, which can be attributed to Arg → Ala replacement in these peptides. Although, in a few cases, $z = +5$ precursor ion have been selected for MS/MS, such spectra did not contain sufficient fragment ion peaks that could yield good sequence coverage. Therefore, the MS/MS spectra acquired on the precursor ion charge states, $z = +2$, +3 and + 4 only have been utilized for interpretation and understanding of the fragmentation patterns for the synthetic mutant peptides, even though our main objective was to focus on the CID MS/MS of precursor ion charge states, $+3 \leq z \leq +6$ (*vide supra*). We observed that the ESI CID MS/MS patterns and the sequence coverage of every Arg → Ala mutant peptide were better than their respective standard peptides (Scheme 4, Supplementary Schemes S3 and S5; Table 2, Supplementary Table S1). The carbamidomethyl Insulin-B chain did



Scheme 6. Overview of the fragmentation pathways observed from the ESI CID MS/MS (Q-TOF) of Arg-Mod model peptides, whose lengths are in the range: 25-30 a.a.r..

not fragment properly by applying Ramped CE during CID MS/MS, whereas the [R22A]-Insulin B-chain dissociated almost completely giving a very good fragment ion yield. This suggests that the R22 does indeed have a significant impact in influencing the extent of CID of Insulin B-chain. Contrastingly, in the cases of Glucagon and Melittin standards, Arg did not seem to affect the extent of their respective CID, since the standards themselves fragmented very well upon CID by Ramped CE.

4.1. Effects of Asp, Glu, His and Pro in the MS/MS spectrum of medium-sized peptides

Enhanced fragmentation at certain peptide bonds such as C-termini of acidic a.a. residues (aspartic acid (Asp) and glutamic acid (Glu)), N-terminus of proline (Pro) and C-terminus of histidine (His) have been well documented [31–33]. However, it needs to be realized that these have been observed predominantly on the shorter length peptides (which includes tryptic peptides as well), viz., less than 20–25 a.a.r., whose precursor ion charge states are, $+1 \leq z \leq +3$, as investigated by CID MS/MS carried out in an ion trap.

In this CID MS/MS study by Q-TOF, although we do observe peaks with good intensities arising from the fragmentation at C-terminus of Asp and Glu, those fragment ion peaks are rather not very prominent, even after modifying the sidechain guanidine group of Arg with CHD or PG. (see Table 4 & Supplementary Table S3). We did not find any fragment ion peak arising due to fragmentation at C-terminus of His in all the model peptides studied here, irrespective of chemical modification of sidechain guanido group of Arg or substituting Arg → Ala. Melittin does not contain any Asp, Glu and His; and, more pronounced proline effect was noticed in the case of MS/MS of Melittin, whereby the intensity of $[y_{13}]^{2+}$ was the highest peak and the abundance of $[y_{13}]^{3+}$ was found to be 50% of the intensity of $[y_{13}]^{2+}$, in the MS/MS spectrum of $z = +4$ precursor ion. Proline effect was also conspicuous in the MS/MS of Insulin B-chain#, but the intensity of y_3 was not the most intense fragment ion peak; the signal corresponding to $[y_{12}]^{2+}$ (peptide bond between Val-18 and Cys#-19) was the highest peak in the CID spectrum of $z = +5$ precursor ion of Insulin B-chain# (Table 4). In the case of MS/MS of mutant peptides of Insulin B-chain and Melittin, fragmentation at N-terminus of Pro was the most facile process, as inferred from the respective fragment ion peaks' intensity values. This indicates that the absence of Arg residues in the sequences of mutant peptides is probably the reason for the clear observation of 'proline effect', which was not that conspicuous in the MS/MS spectra of these two standard peptides.

Therefore, this study unveils that the effects due to Asp, Glu and His on the CID MS/MS behavior are perhaps not more pronounced, which might be attributed to the size and/or electronic properties (charge) of the peptides that have been chosen in this investigation. Perhaps, the three-dimensional structure of the precursor ions in the gas phase or vacuum also has an important role in influencing the fragmentation events. The three-dimensional structures of medium-sized peptides are rather more dynamic (in the gas phase) encompassing various kinds of folded and unfolded conformations, which might not permit facile protonation of peptide backbone or may not facilitate proper collisions with the neutral gas, resulting in inadequate yield of fragment ions. In contrast, the shorter peptides are more unfolded or linear, with which several collisional events are possible that could trigger the cleavages of all of their backbone peptide bonds.

Further, we would like to reiterate (as already mentioned; *vide supra*) that this is also a study having relevance with MD proteomics because of two reasons: (1) sequence and (2) length (size) of the medium-sized model peptides chosen herein. Since, trypsin is not a preferred protease in MD proteomics [1], the sequences of the medium-sized peptides studied herein can be apt representative models for MD proteomics. Secondly, the typical sizes/lengths of proteolytic peptides that are investigated in any MD proteomics, are greater than 25 a.a.r, which are capable of adopting ESI charge states, $z > +3$; and the peptides studied herein also have the same attributes.

5. Conclusions

Comparative analysis of MS/MS spectra of different types of Arg-Mod peptides revealed that CHD-modified peptides provide better fragment ion yield than the respective PG-modified peptides. However, the extent of fragmentation and the resulting sequence coverage of the standard peptides and the respective CHD-Mod peptides remains more or less the same. Thus, the outcome of this study indicates that some other chemical reagent that can specifically modify the sidechain guanidine group may be attempted with the aim of not only to minimize the basicity of the guanidine moiety, but also to enhance the fragmentation (CID) propensity, which can provide better sequence coverage, especially for 'medium-sized peptides'.

The CID MS/MS of Arg → Ala mutant peptides yielded better

Table 4
Intensities of b- and y-ions in terms of percentage (%)§ arising from the fragmentation of certain specific peptide bonds, as observed in the MS/MS spectra of Standard, CHD-Mod, PG-Mod of Insulin B-chain# and Mutant peptide [R22A] of Insulin B-chain.

Peptide	Position	b- ions					y- ions				
			Std (z = +5)	CHD-Mod (z = +5)	PG-Mod (z = +5)	Mutant (z = +3)		Std (z = +5)	CHD-Mod (z = +5)	PG-Mod (z = +5)	Mutant (z = +3)
Insulin B-chain#	C-term. of His −5	b_5	✗	✗	✗	✗	y_{25}	✗	✗	✗	✗
	C-term. of His −10	b_{10}	9.01	8.4	10.2	3.24	y_{20}	✗	✗	✗	✗
	C-term. of Glu −13	b_{13}	12.69	16.3	20.5	11.55	y_{17}	2.9	✗	✗	✗
	C-term. of Glu −21	b_{21}	✗	✗	✗	11.47	y_9	✗	✗	✗	15.84
	N-term. of Pro −28	b_{27}	✗	✗	✗	21.12	y_3	26.5	24.8	34.1	100
Fragment Ion with Highest Peak Intensity§					$[b_{15}]^{2+}$ *{Leu$_{15}$ - Tyr$_{16}$}*			$[y_{12}]^{2+}$ *{Val$_{18}$ - Cys#$_{19}$}*	$[y_{12}]^{2+}$ *{Val$_{18}$ - Cys#$_{19}$}*		y_3 *{Thr$_{27}$ - Pro$_{28}$}*

§ The percentage (%) values shown above have been calculated with reference to the **'fragment ion having highest intensity'** in the respective MS/MS spectrum that are indicated in the above Table with §. In most of the MS/MS spectra analyzed in this study, the 'immonium ion' peak had the highest intensity.

sequence coverage than their respective standard peptides, suggesting that lysines and histidines do not seem to have a greater impact on the extent of fragmentation of these peptides (Scheme 4, Supplementary Schemes S3 and S5). Hence, the MS/MS of Arg → Ala mutant peptides have not only aided in clarifying the role of arginine in impacting the CID MS/MS characteristics, but also has enabled in unraveling the role of the other two basic amino acids: lysine and histidine, especially in the case of peptides of lengths in the range: 25–30 a.a.r. Further, the 'positions of arginines in the primary structure' also play a very critical role in impacting the peptide's dissociation behavior, which might be of worth to be pursued in future. Also, careful optimization of CID MS/MS conditions by suitably varying slope(s) for Ramped CE is imperative.

Altogether, this study shows that Arg can impact the CID MS/MS of the medium-sized peptides to a larger extent than the CID MS/MS of the shorter peptides. And, the results obtained in this study may be useful to understand the tandem mass spectral behavior of certain class of medium-sized natural bioactive peptides, which can be obtained in the sources such as snake venoms, spider toxins, scorpion venoms, conotoxins [34,35]. On a final note, to the best of our knowledge, this is perhaps the first ESI Q-TOF CID MS/MS studies carried out on 'medium-sized' peptides that are 25–30 amino acid residues long, wherein CID is carried out in a hexapole collision cell.

CRediT authorship contribution statement

Pandi Boomathi Pandeswari: Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualization. **Varatharajan Sabareesh:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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