

## Low Molecular Weight Protamine (LMWP) as Nontoxic Heparin/Low Molecular Weight Heparin Antidote (I): Preparation and Characterization

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**ABSTRACT** Low molecular weight protamine (LMWP) appears to be a promising solution for heparin neutralization without the protamine-associated catastrophic toxic effects. The feasibility of this hypothesis was proven previously by using a peptide mixture produced from proteolytic digestion of protamine. To further examine the utility of this compound as an ultimate nontoxic protamine substitute, detailed studies on the purification and characterization of LMWP including the precise amino acid sequence, structure-function relationship, and possible mechanism were conducted. A number of LMWP fragments, composed of highly cationic peptides with molecular weights ranging from 700 to 1900 d, were prepared by digestion of native protamine with the protease thermolysin. These fragments were fractionated using a heparin affinity chromatography, and their relative binding strengths toward heparin were elucidated. Five distinct fractions were eluted at NaCl concentration ranging from 0.4 to 1.0 M and were denoted as TDSP1 to TDSP5, in increasing order of eluting ionic strength. Among these 5 fractions, TDSP4 and TDSP5 contained 3 LMWP peptide fragments, and they were found to retain the complete heparin-neutralizing function of protamine. By using a peptide mass spectrometry (MS) fingerprint mapping technique, the amino acid sequences of the microheterogeneous LMWP fragments in all these 5 elution fractions were readily identified. A typical structural scaffold made by arginine clusters in the middle and nonarginine residues at the N-terminal of the peptide sequence was observed for all these LMWP fragments. By aligning the sequences with the potency in heparin neutralization of these LMWP fragments, it was found that retention of potency similar to that of protamine required the presence of at least 2 arginine clusters in the LMWP fragments;

such as the sequence of VSRRRRRRGRRRR seen in the most potent LMWP fraction-TDSP5. The above finding was further validated by using a synthetic LMWP analogue-CRRRRRRR-and it was found that its heparin-neutralizing ability was increased by changing from a monomeric to a dimeric structure of this analogue peptide. Based on these results, the structural requirement for a compound to function as an effective heparin antidote and the possible mechanism involved in heparin neutralization were established.

**KeyWords:** Heparin/LMWH neutralization; Protamine toxicity; LMWP peptide sequences; MS fingerprint mapping; Mechanism of heparin neutralization

### INTRODUCTION

Extracorporeal blood circulation (ECBC) has become one of the most widely used medical procedures today (1). It is employed in clinical situations such as kidney dialysis and cardiopulmonary bypass. Essentially, ECBC operations require systemic anticoagulation to prevent clotting within ECBC devices, as well as reversal of the anticoagulation at the conclusion of the procedures to alleviate bleeding risks; heparin and protamine are the 2 ubiquitous drugs used to accommodate these purposes. Their combined use, however, has been implicated as the major cause of morbidity and mortality in such procedures (2). Although systemic administration of heparin results in a high incidence of bleeding (3,4), the use of protamine for heparin reversal at times leads to adverse effects ranging from mild hypotension to fatal cardiac arrest (5-7). There is no real alternative for reducing heparin-induced bleeding risks without inflicting patients with protamine-induced toxic effects.

As with other nonhuman protein drugs, protamine could elicit undesired immunologic responses,

namely protamine allergy. In an attempt to alleviate this unwanted protamine immunotoxicity, we previously proposed the development of a low molecular weight protamine (LMWP) fragment as a possible protamine substitute (8). The design of such an LMWP analogue was based on the hypothesis that a chain-shortened protamine fragment containing the heparin-neutralizing domain would still be an effective heparin antidote and yet be devoid of the toxic effects of protamine. In a previous investigation (8), an 1100-d LMWP peptide mixture, prepared by enzymatic digestion of native protamine, was found to retain a high level of heparin neutralizing ability and yet exhibit a significantly reduced level of immunogenicity (ie, the ability to induce the production of antibodies) and antigenicity (ie, crossreactivity toward antiprotamine antibodies), which are the 2 principal events of protamine-induced immunotoxicity. In a continuous effort to develop this nontoxic protamine substitute, we elucidated the peptide sequence, structure-efficacy relationship in heparin neutralization, and toxicity of these LMWP compounds.

Protamine represents a protein family consisting of several small, strongly basic proteins expressed in spermatogenesis. Owing to the presence of only minor sequence differences in these proteins, purification of protamine to homogeneity often proves to be rather difficult (9). Indeed, the presence of microheterogeneity in protamine sequence, which is speculated to result in differences in the immunogenic potential, has been regarded as the major obstacle in establishing the structure-function relationship and assessing the potential toxicity for protamine (10). The LMWP compounds previously developed in our laboratory also inherited such a difficulty because they were heterogeneous in both amino acid composition and sequence (8).

This 3-paper sequence describes our research on developing and testing the LMWP compounds. In this first paper, several LMWP fragments, prepared by enzymatic digestion of native protamine, were prepared and purified using a heparin affinity chromatography. The precise amino acid sequence of each of these LMWP fragments was identified by using a unique mass-mapping technique based on information obtained from matrix assisted laser desorption - time of flight (MALDI-TOF) mass spectroscopic studies. Correlating the sequence

information with the biological functions of these LMWP compounds leads to the structure-function relationship for such protamine-like compounds and a possible mechanism in their action in heparin neutralization. To provide further support of this mechanism, an octapeptide, CRRRRRRR (CR<sub>7</sub>), was synthesized in both monomeric and dimeric forms and tested in its ability to neutralize heparin.

## MATERIALS AND METHODS

### *Materials*

Protamine sulfate (salmine, Grade X), thermolysin (EC 3.4.24.4), trifluoroacetic acid, EDTA, CaCl<sub>2</sub>, and phosphate buffered saline (PBS) solution-ready tablets were purchased from Sigma Chemical Co (St Louis, MO). The HiTrap heparin affinity column was obtained from Pharmacia Biotech Inc (Piscataway, NJ). Porcine intestine heparin (167 IU/mg) was purchased from Pharmacia Hepar Inc (Franklin, OH). Freshly frozen human plasma in citrate was obtained from the American Red Cross in Detroit, MI. Acetonitrile was high pressure liquid chromatography (HPLC) grade, and all aqueous solutions were prepared using distilled and deionized water (ddH<sub>2</sub>O).

### *Preparation of LMWP*

The enzymatic method employed for the preparation of LMWP was described in a previous publication (8). In brief, thermolysin and protamine were mixed in a 1:100 ratio in the PBS solution containing 20 mM CaCl<sub>2</sub>. The reaction mixture was incubated for 30 minutes at room temperature, followed by the addition of EDTA (50 mM) to quench the protease activity. Thermolysin was removed by ultrafiltration using a YM3 membrane (MW Cutoff: 3000 d); the filtrate was then subject to lyophilization. The lyophilized LMWP preparation was stored at -20°C before use.

### *Fractionation of LMWP*

The LMWP preparation was fractionated using a heparin affinity chromatography. A HiTrap heparin column (1 mL) was installed on an Alltech HPLC system (Deerfield, IL) equipped with a Poly Ether Ether Ketone (PEEK) sample loop (1 mL), dual 526 HPLC pumps, a 200 ultraviolet/visible (UV/VIS) detector, and a "PeakSimple for Windows" serial data system. The lyophilized LMWP from the above preparation was dissolved in ddH<sub>2</sub>O to a

concentration of 1 mg/mL, and 1 mL of the solution was injected onto the column. Peptide fractions were separated by using a linear NaCl gradient prepared by mixing solutions of PBS and 2 M NaCl. The column was eluted at a flow rate of 1 mL/min, whereas the NaCl gradient was increased at a rate of 50 mM/min. The elution of the peptide fractions was monitored at 215 nm. A total of 5 peptide peaks, denoted TDSP 1-5 according to the order of elution, was observed. These fractions were collected, desalted, and then lyophilized. Concentration of the peptide in each fraction was quantified at 215 nm using the absorbance of a known concentration of protamine as the reference. All fractions were stored at -20°C until their use.

#### ***Biological Activity of LMWP Measured by the Anti-Xa Assay***

The ACCUCOLOR heparin kit was purchased from Sigma Chemical Co. (St. Louis, MO) and used to determine the neutralization of the anti-Xa activity of heparin by the LMWP fractions. In brief, 20 µL of heparin in human plasma (0.6 U/mL) were mixed with 75 µL of human antithrombin III (0.1 U/mL) at 37°C. Following 2 minutes of incubation, 75 µL of bovine factor Xa (0.24 U/mL), 75 µL of the Xa substrate (1.9 mmol/mL), and 5 µL of protamine (or LMWP) were added. After 10 minutes of incubation, the absorbance in the solution was measured at 405 nm. The heparin-neutralizing ability of protamine (or LMWP) was proportional to the absorbance increase at 405 nm.

#### ***Sequence Identification of Protamine Components***

Major components of protamine were isolated and purified using a reverse-phase HPLC (RP-HPLC). The RP column employed was a 4.6 × 250-mm, 5 µm MacroSphere (Alltech) C4 column. Similar chromatographic procedures described previously were followed. In brief, protamine in PBS solution was applied onto the RP column equilibrated with 0.1% aqueous trifluoroacetic acid and eluted using a shallow gradient of acetonitrile (10%-20% over 90 minutes). The flow rate was set at 0.8 mL/min, and the elution was monitored at 215 nm. Fractions were collected individually and were then subject to amino acid sequence analysis and mass spectrometric analysis.

#### ***MALDI-TOF MS analyses and Sequence Identification of LMWP Fractions by Mass Mapping***

MALDI-TOF MS analyses of the isolated LMWP fractions were performed by the Protein and Carbohydrate Research Center at the University of Michigan using a Vestec-2000 Laser Tec (Houston, TX) research laser desorption linear time of flight mass spectrometer.

The peptide sequences in the isolated LMWP fractions were identified by the mass mapping technique. First, information of the amino acid sequence of the 4 homogeneous protamine fractions obtained above was analyzed by Prophet software (BBN Systems and Technologies, Cambridge, MA) to generate a theoretical peptide sequence map of the thermolysin-digested protamine. The molecular masses of these hypothetical peptides were then matched manually with those of the LMWP fractions acquired by the MALDI-TOF MS analysis. An identical match in mass would provide the LMWP fraction with the identified sequences of the matched hypothetical peptides.

#### ***Synthesis of Analogous Peptides for Study of the Neutralization Mechanism***

A CR<sub>7</sub> peptide that is analogous in composition to LMWP was synthesized in-house using the standard (9-fluorenylmethyl) chloroformate (Fmoc) solid-phase chemistry. This peptide was then purified to homogeneity by using a semipreparative (1 X 25 cm) C18 RP-HPLC column eluted with 0.1% trifluoroacetic acid (TFA) containing a linear gradient of acetonitrile (5%-30%). The purified peptide was lyophilized and stored at -20°C before use.

A dimeric peptide was also prepared by crosslinking the above monomeric peptide in Tris HCl (pH 7.5) at 37°C for 24 hours. The dimeric peptide was purified using a heparin affinity chromatography. Both the monomeric and dimeric peptides were then subject to MS analysis.

## **RESULTS AND DISCUSSION**

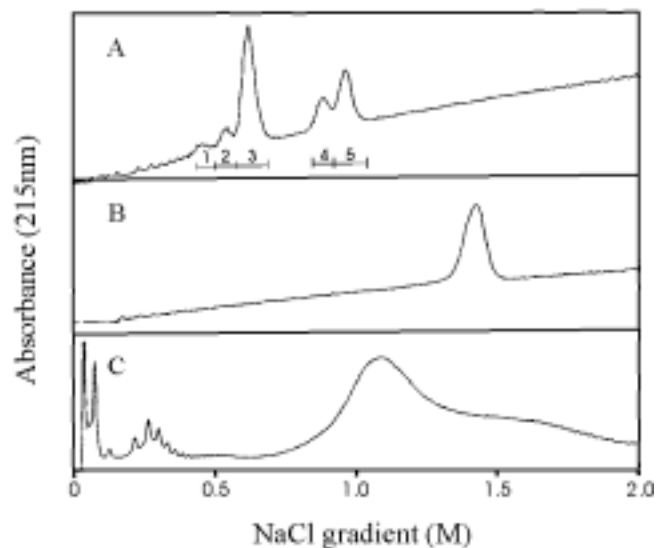
#### ***Preparation and Fractionation of LMWP***

Protamine is a highly basic protein with nearly 67% of its composition in the form of arginine residues. The presence of this high arginine content precluded trypsin or trypsin-like protease from being used as

the digesting enzyme when preparing LMWP from protamine. Such proteases would catalyze the hydrolysis of the arginyl-arginyl bond, leading to an excessive and uncontrollable protamine digestion. To reserve the structural integrity of the arginine sequence, thermolysin was selected. It should be noted that, besides thermolysin, other protease such as ficin (EC 3.4.22.3) and elastase (EC 3.4.21.36) could also be used in digestion of protamine at the nonarginyl bond. These proteases, however, were found to yield LMWP fragments with significantly less heparin-neutralizing ability (data not shown) and were therefore abandoned.

Affinity chromatography based on a heparin column was selected to purify the LMWP preparation. As shown in Figure 1, the thermolysin-digested protamine was fractionated into 5 distinct fractions by the heparin column. These 5 LMWP fractions, eluted at a linear NaCl gradient ranging from 0.4 to 1.0 M, were denoted TDSP1-5 according to their orders of elution (Table 1). Among these fractions, TDSP3 was the primary component that composed more than 50% of the original protein mass based on the absorbance at 215 nm. However, TDSP4 and TDSP5 were the 2 fractions that exhibited the highest degree of heparin affinity. TDSP3, TDSP4, and TDSP5 fractions were therefore chosen for subsequent studies.

It is now understood that protamine neutralizes heparin because of its stronger affinity to heparin than antithrombin III (AT III), thereby dissociating AT III from binding to heparin (11). The LMWP fragments, which were derived from protamine, therefore should presumably follow the same mechanism in heparin neutralization. Thus, the affinity to heparin of these LMWP fractions might serve as an indicator of their ability to neutralize heparin. As seen in Figure 1, AT III was eluted between 0.75 and 1.3 M NaCl with the peak at 1.05 M (Figure 1C), whereas protamine was found to elute between 1.3 and 1.5 M NaCl (Figure 1B). The LMWP fractions appeared to lose their binding affinity to heparin after thermolysin digestion—only TDSP4 and TDSP5 possessed the heparin affinity comparable to that of AT III (Figure 1A). These results suggested that TDSP4 and TDSP5 were probably the only 2 LMWP fractions possessing sufficient heparin-neutralizing ability. For



**Figure 1** Chromatograms from a heparin affinity chromatography of (A) low molecular weight protamine; (B) protamine; and (C) antithrombin III.

**Table 1. Properties of Protamine and Low Molecular Weight Protamine (LMWP)**

Name	NaCl <sup>a</sup> Concentration	Components <sup>b</sup>	MW <sup>c</sup> (Daltons)
Protamine	~1.4 M	4	~4200
TDSP5	0.89-0.97 M	1	1879
TDSP4	0.80-0.89 M	2	1695, 1723
AT III	0.75-1.3 M	-	-
TDSP3	0.57-0.69 M	≥6	1100-1400
TDSP2	0.50-0.57 M	≥5	900-1500
TDSP1	0.40-0.49 M	≥3	600-1500

<sup>a</sup>Elution concentration from a heparin affinity column;

<sup>b</sup>Determined by using the reverse-phase high performance liquid chromatography method;

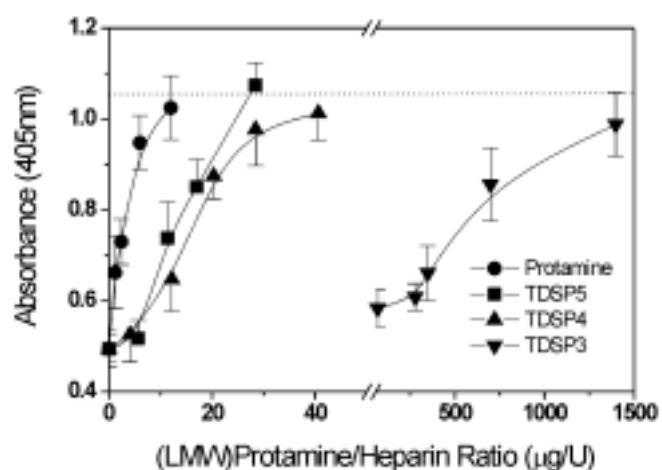
<sup>c</sup>Determined by using the matrix assisted laser desorption - time of flight mass spectrometric analysis.

comparison purposes, all these properties were summarized in Table 1.

### ***Efficacy in Heparin Neutralization of the LMWP Fractions***

To further validate these findings, the efficacy of these LMWP fractions to neutralize heparin was examined using the anti-Xa chromogenic assay. As shown in Figure 2, despite being less potent than protamine, TDSP4 and TDSP5 nevertheless competed effectively with AT III in their binding to heparin. Statistically, a complete neutralization of heparin anti-Xa activity (ie, the dotted line in Figure 2) was achieved by all these 3 compounds of protamine, TDSP5, and TDSP4 at doses of 12, 27,

and 40 µg/U heparin, respectively. On the other hand, the dose of TDSP3 needed to reach the absorbance plateau (ie, 100% heparin neutralization) was substantially higher; up to 1400 µg of TDSP3 was required to achieve only 75% neutralization of 1 U heparin. These results were consistent with data in Table 1, which showed that TDSP3 possessed a much weaker heparin affinity relative to TDSP4 and TDSP5. In conclusion, both TDSP4 and TDSP5 appeared to preserve the heparin-neutralizing domain in protamine, although the required dose for complete heparin neutralization was about 2 to 3 times higher than that of protamine.



**Figure 2 Neutralization of heparin by protamine and low molecular weight protamine as measured by the anti-Xa chromogenic assay. The dashed line represents the control (ie, 100% neutralization).**

**Table 2. Sequences Identified by the Mass-Mapping Approach for Peptides in Low Molecular Weight Protamine (LMWP) Fractions**

Sequence	Protamine* Fractions	LMWP# Fractions	Mass (calc. <sup>a</sup> /obs. <sup>b</sup> )
PRRRR	peak2,3,&4	TDSP1	739.9/740.8
PRRRRR	peak1	TDSP2	896.1/896.9
VRRRRRPR	peak2&3	TDSP2&3	1151.4/1152.2
IRRRRRPR	peak1&4	TDSP2&3	1165.4/1166.2
PRRRRSSSRP	peak2	TDSP2	1254.4/1255.0
PRRRRSSSRP	peak3	TDSP3	1323.5/1324.2
PRRRRSSSRP	peak1	TDSP3	1410.6/1411.2
ASRRRRRGRRRR	peak1	TDSP4	1696.0/1695.5
VSRRRRRGRRRR	peak4	TDSP4	1724.0/1723.1
VSRRRRRGRRRR	peak2&3	TDSP5	1880.2/1879.7

<sup>a</sup>Mass was calculated as the average M+1 of the free base;

<sup>b</sup>Determined by using the matrix assisted laser desorption - time of flight mass spectrometric analysis; \*See Figure 3 for information; #See Figure 1 for information.

### **Sequence Analysis and Characterization of the LMWP Fractions**

Traditionally, peptide sequence was determined by a stepwise, chemical degradation of the purified peptide. The success of this approach relied deeply on the purity and the homogeneity of the target peptide. However, with the presence of an extraordinarily high arginine content and the microheterogeneity in amino acid sequence in protamine, a complete purification and sequence analysis of each of the thermolysin-produced LMWP fragments by using conventional means seemed difficult and, indeed, nearly impossible to achieve. Thus, a computer-aided fingerprint matching of fractionated peptide mixture by MALDI-TOF MS analysis appeared to be both a powerful and the only alternative for sequence identification of these LMWP fragments. The MALDI-TOF MS method adopted in our study had already been widely employed in proteomic research (12). To perform this study, native protamine was first resolved into 4 homogeneous fractions using RP-HPLC chromatography (Figure 3A). These 4 fractions were then subjected to mass analysis and amino acid sequence analysis. As seen in Figure 3B, an interchangeable microheterogeneity and variations of amino acids were observed only at certain positions in these isolated protamine fractions. Coincidentally, these determined amino acid sequences were in complete agreement with those reported by Hoffmann et al (10) for protamine from chum salmon. Based on these identified protamine sequences, a hypothetical thermolysin-digested peptide map was constructed using the Prophet computer software. By manually mapping the mass data acquired from the MALDI-TOF MS analysis of the real thermolysin-produced LMWP fractions with those in the hypothetical peptide map, 10 peptide sequences with molecular weight ranging from 700 to 1900 d were identified for the 5 LMWP fractions (Table 2). By aligning them together, as seen in Table 2, one would find that all of these peptides exhibited a remarkable simplicity with regard to the amino acid composition and sequence. Indeed, the typical structure of these peptides was a scaffold made by arginine clusters in the middle and nonarginine residues at the N-terminal. These peptide sequences could easily be combined to regenerate the original protamine sequences, except

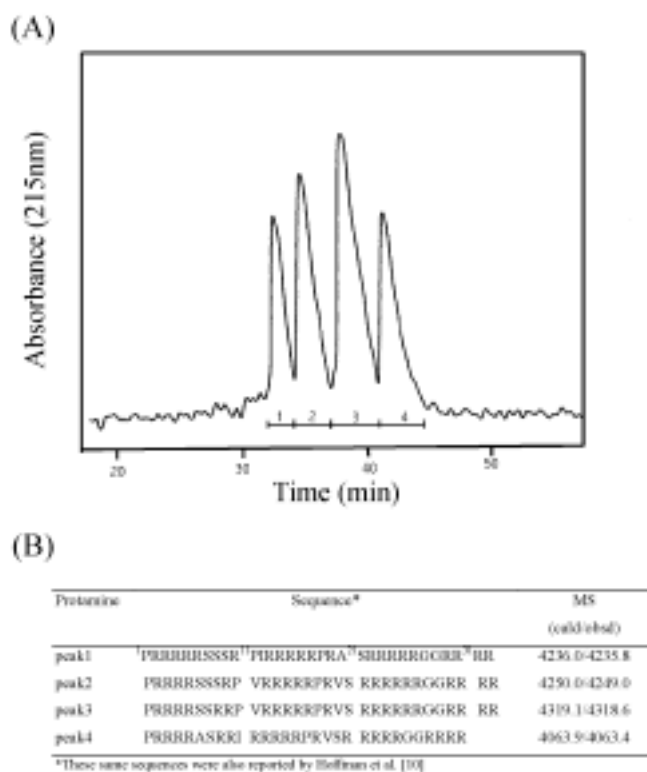
that the ASRR peptide seen in the peak 4 fraction of protamine was somehow lost during the desalting process of the LMWP preparation. In general, the structural attributes of these peptides were in agreement with our previous assumption, which indicated that an intact arginine sequence was essential to retaining the effectiveness in heparin neutralization.

Also shown in Table 2 is the presence of 3 structurally distinct peptides in TDSP4 and TDSP5, the 2 most potent antiheparin fractions. Compared to others, these 3 peptides all possess an additional second arginine cluster. It has been reported in the literature that the binding domains in heparin-binding proteins are those with a high density of positive charges, and their interaction with heparin is predominantly electrostatic (13). Our findings seemed to suggest that a minimal requirement of 2 arginine clusters, each containing 4 to 6 arginine residues, is essential to achieve a binding affinity strong enough to completely neutralize heparin.

### **Possible Mechanism for Heparin Neutralization by LMWP**

As discussed above, 2 arginine clusters seem to be required for LMWP to yield substantial neutralizing ability. To elucidate this mechanism further, CR<sub>7</sub>, containing a single cluster of 7 arginine residues, was synthesized. Owing to the existence of a cysteine residue at the N-terminal of this peptide, a dimer containing 2 such arginine clusters could be readily prepared by the formation of a disulfide bond between the 2 monomers. As shown in Figure 4A, conjugation of the 2 monomers into a dimer significantly increased the binding affinity toward heparin, as reflected by the marked increase in the eluting ionic strength. In addition, the effectiveness in heparin neutralization by the dimer peptide, as measured by the anti-Xa chromogenic assay, was also considerably magnified (Figure 4B). This increased effect in heparin neutralization was particularly evident when the molecular weight difference between the monomer and dimer was taken into consideration. These results strongly implicate that 2 arginine clusters, linked possibly by a Gly-Gly bond as seen in TDSP4 and TDSP5, are probably critical in retaining the complete heparin neutralizing ability. It should be noted, however, that despite being eluted from the heparin column at an ionic strength (ie, 1.4 M NaCl) similar to that of protamine, the dimer peptide nevertheless possessed a weaker heparin neutralizing ability when comparing to protamine (see Figure 4B).

Heparin is a linear polysaccharide made of a disaccharide repeating unit consisting of a  $\alpha$ -D-glucosamine residue alternating with either uronic acid,  $\alpha$ -L-iduronic acid, or  $\beta$ -D-glucuronic acid residue. The chemical structure of heparin, however, is rather complex because of variations in the substitution at the N- and O-sulphate groups, as well as at the N-acetyl groups. Although the anticoagulation function of heparin has been attributed to a specific pentasaccharide sequence responsible for binding to AT III (14), the exact mechanism of heparin neutralization by protamine via the dissociation of AT III from this pentasaccharide sequence remains unclear. A consensus heparin-binding sequence (-X-B-B-X-B-X or X-B-B-B-X-X-B-X-; where X represents a hydrophobic or uncharged amino acid, and B a basic amino acid) was proposed by Cardin and Weintraub



**Figure 3 (A) Reverse-phase high performance liquid chromatography chromatogram of protamine. Flow rate: 1.0 mL/min; temperature: 30°C; column: Alltech C4 (0.46 X 25 cm, 5  $\mu$ m inner diameter). Gradient was prepared by mixing 0.1% trifluoroacetic acid (TFA) with acetonitrile (10%-20% over 90 minutes). Absorbance was monitored at 215 nm. (B) Amino acid sequence and molecular weight of the fractions in protamine.**

(13) based on information of the general sequence present in a large number of heparin-binding proteins. These authors also depicted a model that the basic residues in such a heparin-binding sequence aligned in a special arrangement that could optimize their electrostatic interactions with the acidic groups in heparin. The helical wheel diagram of the protein segments containing the consensus heparin-binding sequences segregated the basic amino acid residues to one face, forming a high density of positive charges that heparin could easily access. This model was later partly confirmed by Lellouch and Lansbury (15) in their study of the peptide sequence required for heparin interaction. Interestingly, the peptide sequences seen in the TDSP1-3 fractions were in good agreement with the consensus heparin-binding sequence in this study, with the presence of only 1 arginine cluster for interaction with heparin. Consequently, our findings with the TDSP4 and TDSP5 fractions suggest that an additional arginine cluster is required to exceed the interaction with heparin from simple binding to the neutralization of its anticoagulant activity.

To test this hypothesis, the efficacy of heparin neutralization by TDSP3, TDSP5, the monomeric and dimeric forms of the synthetic peptide CR<sub>7</sub>, and protamine were evaluated using the anti-Xa assay. The results are summarized in Table 3. It was clear that protamine, which possessed the largest number of arginine clusters (ie, 4), exhibited the highest potency in neutralizing the anti-Xa activity of heparin. On the other hand, TDSP3 and CR<sub>7</sub> monomer, which possessed only 1 arginine cluster, yielded the lowest potency in heparin neutralization. The stronger heparin-neutralizing ability of the CR<sub>7</sub> monomer was most likely the result of a larger amount of arginine residues in the arginine cluster. Doubling the number of the arginine clusters, as seen in the CR<sub>7</sub> dimer and TDSP5, resulted in a remarkable increase in heparin-neutralizing efficacy, ranging from 5 to 80 times that of the CR<sub>7</sub> monomer and TDSP3, respectively. The fact that TDSP5 possessed a slightly higher potency in heparin neutralization and yet a lower total number of arginine residues in the 2 arginine clusters than the CR<sub>7</sub> dimer suggested that the secondary structure of the cationic peptide might also play an important role in heparin neutralization.

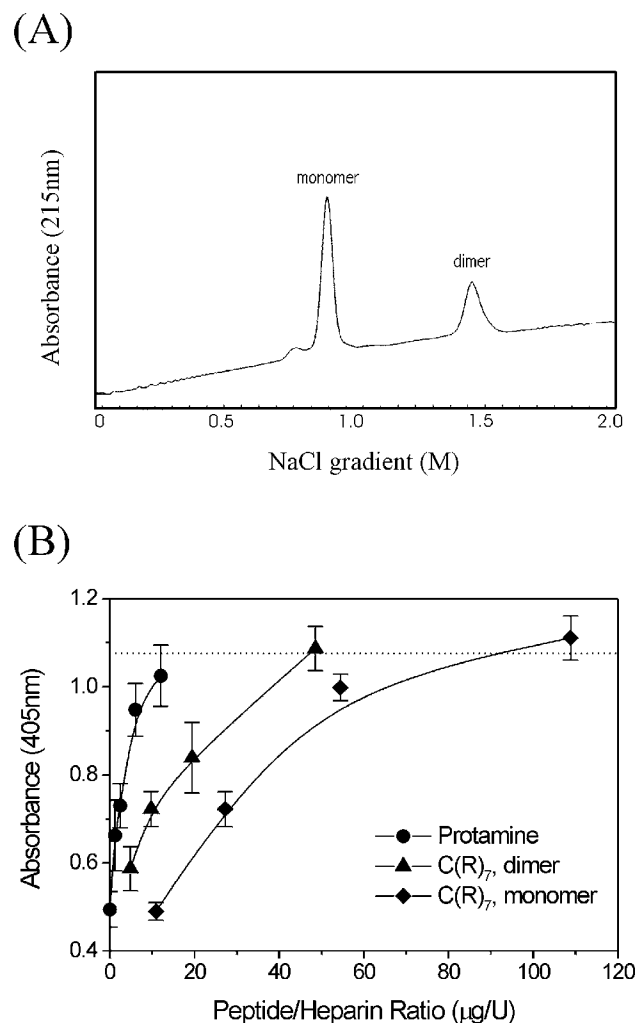


Figure 4 (A) Chromatograms of the CRRRRRRR (CR<sub>7</sub>) monomer and dimer from a heparin affinity column. (B) Heparin neutralization by protamine, CR<sub>7</sub> monomer, and CR<sub>7</sub> dimer as measured by the anti-Xa assay. The dashed line represents the control (ie, 100% neutralization).

Table 3. Efficacy in Heparin Neutralization by Protamine, Low Molecular Weight Protamine Fractions, and CRRRRRRR (CR<sub>7</sub>) Peptides

Compound	Number of Arginine Clusters*	Neutralizing Efficacy (Peptide/Heparin, nmol/U)
Protamine	4	2.9†
TDSP3	1	1000†
TDSP5	2	13.3
Monomer (CR <sub>7</sub> peptide)	1	90.5
Dimer (CR <sub>7</sub> peptide)	2	20.6

\*See Table 2 for information; †Both protamine and TDSP3 are heterogeneous compounds. Efficacy was determined by using an approximate molecular weight of 4200 and 1200 d for protamine and TDSP3, respectively.

Studies using nuclear magnetic resonance spectroscopy (NMR) spectroscopy demonstrated that heparin yielded a well-defined conformation in solution (16). This solution conformation of heparin also displayed the presence of clusters of sulphate groups down each side of the molecule, with a distance of about 17 Å between 2 clusters. Although it is not known yet whether the spacing by 2 glycine residues in TDSP4 and TDSP5 will provide a better matching of their 2 arginine clusters with the 2 sulfate clusters in heparin, our findings nevertheless demonstrate that 2 arginine clusters are the minimal structural requirement to achieve effective heparin neutralization.

## CONCLUSIONS

Two LMWP fractions (termed TDSP4 and TDSP5), derived directly from native protamine by thermolysin digestion and purified to near homogeneity using a heparin affinity column, were found to neutralize heparin. By using a novel mass-mapping approach based on information obtained from MALDI-TOF mass spectra analysis, the precise amino acid sequences of peptides in these 2 LMWP fractions were identified. Correlation of the sequence information with the heparin-neutralizing function of these LMWP peptides and their synthetic analogues leads to the establishment, for the first time, of the apparent structural requirement for a compound to function as an effective heparin antidote as well as a possible mechanism for its action in heparin neutralization. In the next article in this series, an in vitro evaluation of both the efficacy and toxicity of these LMWP compounds will be presented.

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