Developing Protease-resistant Drug Delivery Vehicles
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ABSTRACT

The cell membrane poses a significant physical barrier to the entry of hydrophilic drug molecules into cells. The discovery of cell-penetrating peptides, or CPPs, which are able to translocate across cell membranes by a receptor-independent manner, was a major breakthrough in cellular drug delivery. However, one disadvantage of using CPP in drug delivery is their low metabolic stability. We have discovered that a peptide (alpha-I-gliadin) derived from wheat gluten is cell permeable and is also protease resistant. In this study we have investigated the protease susceptibility of native and modified versions of alpha-I-gliadin and compared it to that of Tat, which is the most commonly used CPP today. Alpha-I-gliadin, its derivatives and Tat were synthesised using Fmoc chemistry and were challenged with gastrointestinal and serum proteases in vivo. Reversed-phase HPLC was used to analyse the digestion products. Native and deamidated alpha-I-gliadin peptides were found to be almost completely resistant to gastrointestinal and serum proteases; in contrast, Tat was readily degraded under the same conditions. Alpha-I-gliadin is a promising candidate for the next generation CPP.

INTRODUCTION

The lipid bilayer of the cell membrane poses a significant physical barrier to the entry of hydrophilic drug molecules into cells. The discovery of cell-penetrating peptides or CPPs, which are able to translocate across cell membranes by a receptor-independent manner, was a major breakthrough in cellular drug delivery. An important factor which determines the pharmaceutical application of CPPs is their metabolic stability. A drug cargo should be carried to its target before the peptide drug delivery vehicle is degraded by proteases. The purpose of this study is to develop novel peptides with improved metabolic stability compared to Tat peptide. Native, deamidated, modified alpha-1-gliadin and Tat were subjected to in vitro digestion by gastrointestinal and serum proteases and the digestion mixtures analysed by reversed-phase high performance liquid chromatography (RP-HPLC) to determine the extent of proteolysis.

MATERIALS AND METHODS

Peptide synthesis
The peptides QLQPFPQPQLPY, QLQPFPQPELPY, RRRPRPQPELPY and YGRKKRQQRQRR were obtained by solid-phase synthesis using 9-fluorenylemethoxycarbonyl (Fmoc) chemistry and purified by semi-preparative RP-HPLC. The identity and purity of the

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peptides were confirmed by electrospray ionisation mass spectrometry (ESI-MS) and analytical RP-HPLC respectively.

In vitro enzymatic digestion
Pepsin was added to a solution of peptide in HCl and incubated at 37°C for 30min. Pepsin was deactivated by the addition of sodium phosphate buffer. Chymotrypsin, trypsin, elastase and carboxypeptidase A were then added to the digestion mixture and incubated at 37°C for two hours. The enzymes were deactivated by heating at 95°C for 10 min. Leucine aminopeptidase was added to a solution of peptide in sodium phosphate buffer and incubated at 37°C for six hours before the reaction was quenched by heating at 95°C for 10 min. The digestion mixture was ultrafiltrated to separate the enzyme from the peptide digestion products and the filtrate collected. The digestion mixtures were analysed by analytical RP-HPLC.

RESULTS

Peptide synthesis
All four peptides were successfully synthesised using Fmoc chemistry. The purity and molecular mass of all the synthesised peptides are summarised in table 1.

Table 1: Purity and molecular mass of synthesised peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Purity (%)</th>
<th>Molecular mass (Da)</th>
<th>Molecular mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native alpha-1-gliadin</td>
<td>QLQPFPQPQLPY</td>
<td>95.5</td>
<td>1455.7</td>
<td>1455.8</td>
</tr>
<tr>
<td>Deamidated alpha-1-gliadin</td>
<td>QLQPFPQPPELPY</td>
<td>96.3</td>
<td>1456.7</td>
<td>1456.6</td>
</tr>
<tr>
<td>Modified alpha-1-gliadin</td>
<td>RRRPRPQPELPY</td>
<td>97.2</td>
<td>1564.8</td>
<td>1565.6</td>
</tr>
<tr>
<td>Tat</td>
<td>YGRKKRRQRRR</td>
<td>98.4</td>
<td>1559.9</td>
<td>1560.5</td>
</tr>
</tbody>
</table>

In vitro digestion by gastrointestinal enzymes
The synthesised native, deamidated and modified alpha-1-gliadin and Tat peptides were incubated with pepsin, followed by trypsin, chymotrypsin, elastase and carboxypeptidase A to simulate gastrointestinal digestion. The results summarised in Table 2 indicate that besides native alpha-1-gliadin peptide, which was previously reported to be resistant to gastrointestinal proteolysis (Hausch et al., 2002), the deamidated and modified alpha-1-gliadin peptides are also highly resistant to digestion by GI enzymes.

In vitro digestion by leucine aminopeptidase
The proteolytic stability of the four peptides in serum was estimated by incubation with the serum protease leucine aminopeptidase. Leucine aminopeptidase was chosen as the representative serum protease as under physiological conditions, aminopeptidases are found to be more active than carboxypeptidases in the serum (Galati et al., 2003). Data from Table 2 show that only the native and deamidated alpha-1-gliadin peptides exhibited limited resistance to proteolysis by leucine aminopeptidase. Both the modified alpha-1-gliadin and Tat peptides were completely broken down. For both the native and deamidated alpha-1-gliadin peptides,
only the pyroglutamate form was left intact after incubation with leucine aminopeptidase for six hours, while the peak due to the parent peptide disappeared completely.

Table 2: Extent of in vitro digestion of peptides by proteases

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Percentage of parent peptide digested by gastrointestinal enzymes</th>
<th>Percentage of parent peptide digested by leucine aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native alpha-1-gliadin</td>
<td>QLQPFPQPQLPY</td>
<td>8</td>
<td>86</td>
</tr>
<tr>
<td>Deamidated alpha-1-gliadin</td>
<td>QLQPFPQPELPY</td>
<td>11</td>
<td>85</td>
</tr>
<tr>
<td>Modified alpha-1-gliadin</td>
<td>RRRPRQPELPY</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Tat</td>
<td>YGRKKRRQRRR</td>
<td>98</td>
<td>100</td>
</tr>
</tbody>
</table>

**DISCUSSION**

As expected, Tat was found to be vulnerable to gastrointestinal digestion due to its multiple arginine and lysine residues which render it susceptible to tryptic cleavage. None of the parent peptide remained on incubation with leucine aminopeptidase. This revealed the possible metabolic instability of Tat peptide in the blood plasma. Human blood plasma contains trypsin-like proteases like plasmin which has similar cleavage specificity to trypsin. Tat peptide is therefore highly unsuitable as a drug delivery vehicle in intravenous and oral administration preparations.

The native and deamidated alpha-1-gliadin peptides were found to be highly resistant to proteolysis by gastrointestinal enzymes, in accordance with previous similar studies (Hausch et al., 2002; Piper et al, 2004). In contrast, the proteolytic degradation patterns for these two peptides differed greatly on incubation with leucine aminopeptidase. This was unexpected as the only difference between the two peptides was at the 9th residue from the N-terminus. Nevertheless, both peptides displayed similar resistance to digestion by leucine aminopeptidase. This similarity was likely to be due to the presence of the aminopeptidase-resistant pyroglutamate forms of the peptides. Hence, the native and deamidated alpha-1-gliadin peptides are suitable as protease-resistant drug delivery vehicles in intravenous and oral administrations.

The modified alpha-1-gliadin peptide was surprisingly found to be fairly resistant to gastrointestinal proteolysis. This would have rendered it suitable for oral administration. However, it was found to be completely digested by leucine aminopeptidase after six hours of incubation, thus rendering it unsuitable for either intravenous or oral administration.

A more complete and accurate analysis of the digestion mixtures could have been performed by LC-MS. This will allow for the easy identification of the parent peptide peaks instead of comparing the retention volumes of peaks with those in the blank runs. Slight changes in retention volume may occur between runs due to variation in operating conditions, which makes the latter method of identifying the parent peptide peak highly unreliable. In addition, LC-MS facilitates the identification of the cleavage products and therefore the cleavage sites in the original peptide. This knowledge can be applied to the sequence modification of the alpha-1-gliadin peptide in order to design peptides with even greater protease stability.
There is plenty of room for future work in this study. Incubation with leucine aminopeptidase provides only a rough approximation to the serum stability of the peptides. The stability of the alpha-1-gliadin peptides in human serum can be tested to determine their viability for pharmacological applications. The time-course of the proteolysis of the peptides could have been followed by withdrawing aliquots of the digestion mixtures at certain time periods and quenching them before performing analysis by HPLC. This will help in the estimation of the half life of the peptides in the presence of the enzymes, and thus provide a better picture of their proteolytic stability. A final test of the viability of oral administration of the peptides can be performed with brush border membrane (BBM) preparations as detailed by Hausch et al. (2002). Finally, cell penetration studies should be made on the three alpha-1-gliadin peptides to determine their cell penetration propensity.

The native and deamidated alpha-1-gliadin peptides have been found to be much more resistant to degradation by gastrointestinal and serum proteases than the most well-known and studied CPP, Tat. This bodes well for their potential application as drug delivery vehicles. This study has also shown that the N-terminal pyroglutamylation of peptides with an N-terminal glutamine residue enhances their stability to digestion by aminopeptidases. The protease resistance of the native and deamidated alpha-1-gliadin peptides can be increased easily by heating them at $95^\circ$C, which increases the rate of formation of the pyroglutamate form drastically. Pyroglutamylation is preferred to other forms of N-terminal modification such as PEGylation as the pyroglutamate form is likely to have very similar chemical and physical properties with the parent peptide, thus retaining its cell-penetration propensity.

It has been shown that the native and deamidated alpha-1-gliadin peptides are significantly more resistant to degradation by gastrointestinal enzymes and the serum enzyme, leucine aminopeptidase than the CPP, Tat. The next steps are to evaluate the cell-penetration propensity of these two peptides and to extend the protease study to human serum and brush border membrane preparations. The results of these studies will confirm the status of alpha-1-gliadin peptide as a protease-resistant drug delivery vehicle with important pharmacological applications.

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REFERENCES

