Short Communication

Affinity precipitation of trypsin with soybean trypsin inhibitor linked Eudragit S-100

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Received 28 April 1994; accepted 13 July 1994

Abstract

Soybean trypsin inhibitor linked to Eudragit S-100 was used for the affinity precipitation of trypsin. Polymer and ligand concentrations used in conjugate preparation showed remarkable effect on the trypsin recovery. Trypsin precipitation efficiency amounted to 89% and recovery was 74%. The final purification of relatively crude commercial trypsin resulted in 1.85-fold purification. The SDS-PAGE analysis indicated significant purification. The precipitated enzyme activity was around 96% and recovered enzyme activity was 83%.

Keywords: Affinity precipitation; Benzamidine; Eudragit S-100; Soybean trypsin inhibitor; Trypsin

1. Introduction

In recent years, affinity precipitation has emerged as a useful approach for reducing the number of steps in the purification protocols for proteins (Pecs et al., 1991; Gupta and Mattiasson, 1992; Kamihara et al., 1992). Choosing STI-trypsin as a model affinity pair like many other previous workers (Luong et al., 1988; Senstad and Mattiasson, 1989; Pecs et al., 1991), we report here affinity precipitation of trypsin from a commercial crude preparation with the conjugate of STI with Eudragit S-100 which is an enteric coating methacrylate polymer (Kamihara et al., 1992).

2. Materials and methods

Materials

Eudragit S-100 was a gift from Professor Bo Mattiason of the Department of Biotechnology, Chemical Centre Lund, Sweden. Crude bovine pancreatic trypsin was purchased from Sigma Research Laboratories, India. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, USA.

Affinity precipitation of trypsin with Eudragit-STI conjugate

A stock solution (2%) of Eudragit was prepared in phosphate buffer (0.01 M, pH 7.2) containing 0.15 M NaCl. The soybean trypsin inhibitor (STI) was conjugated to Eudragit S-100 by using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC) (Kamihara et al.,

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After optimization, the conjugate used for the precipitation of trypsin was the one prepared by using a starting STI concentration of 0.1 mg ml\(^{-1}\) and polymer concentration of 1%. Before using the conjugate for trypsin precipitation, it was titrated against various concentrations of trypsin. On the basis of titrant obtained, 3 ml of conjugate were added to 1 ml of trypsin (0.8 mg ml\(^{-1}\) in Tris-HCl, pH 8.0 containing 0.02 M CaCl\(_2\)) and incubated at 25°C for 30 min. The whole mixture was precipitated by decreasing the pH to 4.5 with 0.1 M acetate buffer containing 0.02 M CaCl\(_2\). The precipitate was centrifuged at 12,000 rpm for 15 min and washed twice with 5 ml of 0.01 M acetate buffer (pH 4.5) containing 0.02 M CaCl\(_2\). The precipitation of partially crude commercial trypsin was carried out by adding 300 mg of crude trypsin (5.4 mg protein) to 12 ml of STI-conjugate (starting STI concentration 0.1 mg ml\(^{-1}\)).

The recovery of trypsin was carried out by washing the precipitate with glycine-HCl buffer (0.1 M, pH 2.5) containing 0.5 M NaCl and 0.02 M CaCl\(_2\). This did not give adequate recovery in the case of crude preparation. In that case, elution of trypsin was carried out by dissolving the precipitate in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M CaCl\(_2\). Benzamidine hydrochloride was added to the dissolved precipitate to the final concentration of 350 mM. Trypsin was separated from the dissociated Eudragit-STI by precipitating the latter by lowering the pH to 4.5.

Analytical procedures

Trypsin activity was measured according to the method of Erlanger et al. (1961) using \(N\)-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate.

Protein concentration was determined by dye binding assay (Bradford, 1976) using bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gel was performed according to Hames (1986).

3. Results and discussion

The precipitation behavior of Eudragit-STI conjugate was similar to Eudragit (i.e., nearly quantitative precipitation below the pH in the range of 4.8 to 5.2). The conjugates prepared by varying STI concentration (0.05 mg to 1.0 mg ml\(^{-1}\) of 1% Eudragit solution) showed that in all cases 80% of the protein was bound to the polymer. The trypsin inhibiting activity of the conjugate increased initially but leveled off at STI concentration beyond 0.15 mg ml\(^{-1}\) of the polymer, presumably because STI 'crowded' the matrix.

3.1. Effect of polymer concentration on STI conjugation

The effect of polymer (Eudragit S-100) concentration on STI conjugation was studied. It was observed that at all polymer concentrations studied (0.25–3.0%) the conjugation efficiency of STI to Eudragit remained almost the same (79–83%). These conjugates expressed the same STI activity 58–59% (of activity started with) bound to the polymer. These results show that in the polymer concentration range used, viscosity changes in the reaction medium do not affect conjugation efficiency of EDC protocol.

3.2. Effect of polymer concentration on trypsin precipitation and recovery

Since all the conjugates prepared at different polymer concentrations showed the same expressed STI activity, the same amount of trypsin (0.8 mg per 3 ml of conjugate at STI trypsin activity ratio of 1.5) were used for precipitation.

It was observed that at all polymer concentrations (0.25–3.0%), the trypsin precipitation efficiency of the conjugate remained almost same 79–83% (Table 1). However, the maximum trypsin activity recovered from the precipitated trypsin was 84% at 0.25% Eudragit concentration. The recovered trypsin activity decreased as the polymer concentration was increased. The effect was negligible until 1% Eudragit concentration, but activity recovery decreased significantly (28%) as the polymer concentration was increased to 3%. This is presumably because at higher polymer concentrations, the settling of the Eudragit-STI was difficult and a small increase of unprecipi-
Table 1
Effect of polymer concentration on trypsin precipitation and recovery

<table>
<thead>
<tr>
<th>Polymer concentration (%)</th>
<th>Trypsin precipitated (%)</th>
<th>Trypsin recovered (%)</th>
<th>Protein</th>
<th>Activity *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>82</td>
<td>79</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>0.5</td>
<td>83</td>
<td>80</td>
<td>66</td>
<td>68</td>
</tr>
<tr>
<td>1.0</td>
<td>81</td>
<td>77</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>2.0</td>
<td>79</td>
<td>76</td>
<td>52</td>
<td>64</td>
</tr>
<tr>
<td>3.0</td>
<td>79</td>
<td>75</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

*Activity recovered after filtration. 1 ml of trypsin solution (0.8 mg ml⁻¹) was precipitated separately with 3 ml of conjugate prepared by varying Eudragit concentration (0.25-3.0%).

3.3. Optimization of precipitation and recovery of trypsin

At a polymer concentration of 1% and conjugates prepared with initial STI concentration of 0.1 mg ml⁻¹, efforts were made to increase the precipitation efficiency of trypsin. This was done by adding fresh STI-Eudragit conjugate to the unprecipitated trypsin.

To the supernatant containing some unprecipitated trypsin, the addition of fresh Eudragit-STI conjugate precipitated trypsin at an efficiency of 39%. Further precipitation of the unprecipitated trypsin was again carried out by adding fresh conjugate, which led to 10% precipitation. However, further addition of conjugate could not precipitate the unprecipitated trypsin. In this way a total of 89% of trypsin precipitation was achieved. This also led to higher protein recovery of 85% and activity recovery of 74%.

3.4. Purification of crude trypsin

Encouraged by the above results, a relatively crude trypsin preparation (from bovine pancreas) which was commercially available was purified using a similar approach. The Eudragit-STI conjugate precipitated 68% of the total protein and 96% of enzyme activity. The washings with 0.01

Table 2
Purification of trypsin by affinity precipitation

<table>
<thead>
<tr>
<th>Stages</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹ protein)</th>
<th>Enzyme yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting</td>
<td>5.40</td>
<td>1458</td>
<td>270</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant after precipitation</td>
<td>1.73</td>
<td>58.3</td>
<td>ND</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td>Washings (with 0.01 M acetate buffer pH 4.5 containing 0.02 M CaCl₂)</td>
<td>1.08</td>
<td>0.0</td>
<td>ND</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Eluted sample (with 350 mM benzamidine)</td>
<td>2.43</td>
<td>1210</td>
<td>498</td>
<td>83</td>
<td>1.85</td>
</tr>
</tbody>
</table>

ND, not determined.

Trypsin was precipitated from 300 mg of crude commercial trypsin preparation (protein 5.4 mg) with 12 ml of Eudragit-STI conjugate.
M acetate buffer (pH 4.5) containing 0.02 M CaCl₂ removed 20% protein, but no enzyme activity was detected in the washings.

When the elution with glycine-HCl buffer (0.1 M, pH 2.5) containing 0.5 M NaCl and 0.02 M CaCl₂ was tried for recovery of the trypsin from the precipitate, only 6% of the initial protein could be recovered. Even dissolution of the precipitate in Tris-HCl buffer (0.05 M, pH 8.0 containing 0.02 M CaCl₂), followed by precipitation by addition of glycine-HCl buffer to reach pH 3.0, also did not leave more than 6% of the protein in the supernatant. The use of elution with arginine has been recommended by some workers in similar systems (Male et al., 1987). In the present system elution with arginine (625 mM) did not yield any measurable protein.

Finally, the following method was used for recovery of the trypsin activity. The precipitate containing trypsin was dissolved in Tris-HCl buffer (0.05 M, pH 8.0 containing 0.02 M CaCl₂). Varying amounts of benzamidine, the competitive inhibitor of trypsin (Mares-Guia and Shaw, 1965), were incorporated before precipitating the polymer by addition of 0.2 M acetic acid to adjust the pH to 4.5. The supernatant was examined for protein recovery. As the recovery of the protein was maximum at 350 mM concentration of benzamidine, the supernatant obtained in this case was dialyzed against Tris-HCl buffer (0.05 M, pH 8.0 containing 0.02 M CaCl₂). 83% of the original starting trypsin activity was recovered. The protein recovery was 45% and the fold purification was 1.85 (Table 2). The SDS-PAGE pattern (Fig. 1) showed a single band as compared to the pattern obtained with crude trypsin which showed multiple bands.

It may be interesting to compare results obtained by us with those obtained by other workers with similar systems. Pecs et al. (1991) have described the purification of trypsin from a commercial preparation using affinity precipitation with p-aminobenzamidine bound polymer. Their precipitation efficiencies were 90%, but the recovery was only 50%. They suggested that efficiency could be increased by further optimization. In the case of Nguyen and Luong (1989), who used p-aminobenzamidine coupled to a copolymer of N-isopropyl acrylamide (NIPAM) and N-acryloylsuccinimide (NASI) or glycidyl methacrylate (GMA), the recoveries of the trypsin activity were 74% and 82%, respectively. More recently, Galaev and Mattiasson (1992) have separated trypsin from its mixture with BSA with an activity yield of 54% using affinity precipitation with STI conjugated with a thermoreactive polymer.

Our results are quite comparable with those of other workers and show that process optimization at each step using a commercially available cheap and nontoxic polymer, viz. Eudragit S-100, affinity precipitation could be successfully employed for purification of trypsin and possibly other proteins/enzymes.

Acknowledgements

This work was supported by the Swedish Agency for Research Cooperation with develop-
References


