 Synthesis, physicochemical and immunological properties of oxidized inulin–L-asparaginase bioconjugate

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\begin{abstract}
In recent years several bioconjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. In this study we investigated the effect of conjugation with oxidized inulin on these properties. Inulin (MW about 25 kDa) was oxidized with periodate and was conjugated to the enzyme at molar ratios of oxidized inulin to enzyme between 2:1 and 4:1. Modified L-asparaginase synthesized at ratio of 2:1 had activity of 65% of that of native enzyme and was used in subsequent experiments. The apparent \( K_m \) of glycoconjugate (0.1 \( \times 10^{-4} \) M) was about five times lower than the \( K_m \) of native enzyme (0.57 \( \times 10^{-4} \) M). Modified L-asparaginase had longer half life, higher thermostability and more resistance to trypsin digestion and better reusability after repeated freezing and wider optimum pH range than that of intact L-asparaginase. The modification of L-asparaginase with oxidized inulin at molar ratio of 2:1 resulted in decreasing antibody (IgG) titer and immunogenecity after repeated injection of rabbits with respect to the native L-asparaginase. Taken together, the results of this study indicated that L-asparaginase modification by oxidized inulin results in a new stabilized product with improved biochemical properties which may be employed for medical purposes.
\end{abstract}

1. Introduction

L-asparaginase (L-asparagine amidohydrolase, EC3.5.1.1, L-ASNase) is an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Mathe et al., 1970). It is derived from two bacterial sources; \textit{E. coli} and \textit{Erwinia carotovora} (Verma et al., 2007; Kotzia and Labrou, 2007; Kotzia and Labrou, 2005). This enzyme has been used as a therapeutic agent against acute lymphoblastic leukemia (ALL), since it was discovered that the hamster serum contain a high level of L-ASNase and has the ability to prevent the growth of induced tumors in mice (Clementi, 1922; Kidd, 1953; Zhang et al., 1995). All leukemic cells are unable to synthesize L-asparagine due to the lack of L-asparagine synthetase activity (Asselin et al., 1989) and are depend on circulating L-asparagine. Normal cells are able to synthesize L-asparagine and thus are less affected by L-asparagine depletion after treatment with the L-ASNase (Marlborough et al., 1975). The enzyme also inhibits protein synthesis by L-asparagine hydrolysis. It is believed that treatment with L-ASNase results in apoptosis of leukemic cells due to cell cycle arrest in G1 phase. Products of L-ASNase, specially \( \text{NH}_4^+ \) ion diffuse into the cytosol and modify the pH, which activate signal transduction pathway associated with phosphorylation of substrates and apoptosis (Ueno et al., 1997; Shimizu et al., 1992). It has been reported that there is a requirement of a functional p53 protein for L-asparaginase to produce apoptosis in HL60 promyelocytic leukemia cell lines (Fu et al., 1998).

Main restrictions to the use of L-ASNase in the therapeutic field are several types of allergic side effects due to high immunogenecity (Mathe et al., 1970). Clinically important toxicities include liver dysfunction, pancreatitis, hyperglycemia, coagulopathy and central nervous system dysfunction (Boos, 1997). Furthermore, L-ASNase has low stability in serum and, accordingly, rapid plasma clearance in the blood system due to action of native protease or specific antibody (Soares et al., 2002). Several protocols have been developed to improve the pharmacokinetic properties of L-ASNase with the main aim of increasing the half life, stability and elimination of the immunogenecity. One approach to improve the function and properties of the enzyme is its chemical modification with various kinds of polymers. L-ASNase was successfully modified with natural and artificial soluble and insoluble polymers such as albumin (Poznansky et al., 1982), dextran (Karsakevich et al., 1986), polyethylene glycol (PEG) (Soares et al., 2002), chitosan (Qian et al., 1996), silk fibroin (Zhang et al., 2005) and sericine (Zhang et al., 2005).
Peg-ASNase or pegaspargase is a bioconjugated form of ASNase that has been commercially available since 1994. It has been reported that this modified form of enzyme has improved the immunity, toxicity and the resistance to proteolysis with respect to native l-ASNase. Based on a recent report that shows a possible higher incidence of pancreatitis, pegaspargase should not be routinely substituted for the native enzyme. (Rizzari et al., 2006). Therefore, in recent years attempts have been made by researchers to improve the l-ASNase properties by using new chemical modification protocols.

Inulin, a nondigestible carbohydrate, is a fructan that is found in many plants as a storage carbohydrate and has been part of mans daily diet for several centuries (Carabin and Flamm, 1999). In recent years there has been a spectacular increase in the number of publications relating to the nutritional benefits of inulin in human health, for example in diabetes mellitus, cancer and obesity (Carabin and Flamm, 1999; Kaur and Gupt, 2002). In this study we investigated the effect of conjugation with oxidized inulin on the physiochemical and immunological properties of l-ASNase.

2. Materials and methods

2.1. Materials

Lyophilized, concentrated l-asparaginase in the form of Elspar® with 1000 IU/25 mg was obtained from EUSA Pharma Inc (USA). Inulin from Chicory with molecular weight about 25 kDa were obtained from Merck (Darmstadt, Germany). Asparagine, trypsin, KI, HgCl₂, trichloroacetic acid, bovine serum albumin, Tween 20 and Sephadex G-100 were purchased from Sigma (St. Louis, MO, USA). Trinitrobenzene sulfonic acid and marker of molecular weight were obtained from Fluka Co. Ltd. (Switzerland). Horseradish peroxidase conjugated goat anti rabbit IgG was from PerkinElmer (USA). Serum were obtained from immunized male rabbits after repeated injection of native or modified l-ASNase.

2.2. Animals

The male adult New Zealand White rabbits (about 1.5 kg body weight) were purchased from the experimental animal center at Shiraz Medical University, Shiraz, Iran. Animals were maintained in a clean rodent room and were housed three per cage at a temperature of 28 ± 1 °C under a daily photoperiod of 12-h light/dark cycle. The animals were handled according to the institutional ethical program for the purpose of experiments on animals.

2.3. Oxidation of inulin

Five hundred mg of inulin powder (MW about 25 kDa) and 200 mg potassium periodate were dissolved in 10 ml water pH 8. The mixture was stoppered tightly and maintained on shaker in the dark at room temperature for 24 h. After this time, separation of oxidized inulin from the unreactant potassium periodate was made through the Sephadex G-10 column (1.5 cm × 25 cm), was eluted with distilled water pH 8. The inulin content was estimated by phenol sulfuric acid method (Dubois et al., 1956) and by using glucose as standard. Purified, oxidized inulin was lyophilized and used in the subsequent experiments.

2.4. Preparation and purification of l-ASNase–inulin conjugate

l-ASNase (20 mmol/ml) was dissolved in 3 ml 0.05 M Tris–HCl buffer, pH 9, containing 40 or 80 mmol/ml oxidized inulin powder and 5.0 mg/ml l-asparagine as an active center protector of the enzyme. Initial reactions were carried out at molar ratios of oxidized inulin to protein at 2:1 and 4:1, to determine the optimal conjugation conditions. The reaction was maintained at 37 °C for 48 h. After this time, two-fold molar excess of NaBH₄ per aldehyde groups was added and slightly mixed for 1 h at room temperature. Impurities, precipitates and excessive non reactant oxidized inulin were removed by extensive dialysis against distilled water at 4 °C for 18 h. The separation and purification of modified l-ASNase from native l-ASNase was made through a Sephadex G-100 column (1.5 cm × 40 cm), equilibrated and eluted with 0.05 M Tris–HCl buffer pH 8. The elution sample with highest activity was set as 100% of relative activity. Based on the results of enzyme activity and electrophoresis analysis the molar ratio of 2:1 was then used for all subsequent reactions. Modification and molecular weight of all preparations were confirmed by SDS-PAGE (Laemmli, 1970). The degree of modification of ε-amino groups was estimated by using Trinitrobenzene sulfonate (TNBS) (Habeeb, 1996). The protein concentration was determined by the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951).

2.5. Assay of l-ASNase activity and kinetic

The enzyme activity was determined from the Nessler reagent, as described by Mashburn and Wriston (1963). The ammonia concentration produced in the reaction was determined based on the standard curve previously obtained with ammonium sulfate as a standard. An International Unit (IU) of l-ASNase is defined as the amount of enzyme that catalyzes the production of 1 μmol of ammonia in 1 min at pH 8.5 at 37 °C.

In kinetic analysis, the Michaelis constants (Kₘ) of native and modified l-ASNase were calculated from the Lineweaver–Burk plots of enzyme activity versus asparagine concentrations between 0.2 and 1.0 mM in 0.05 M Tris–HCl, pH 8.5. All experiments were performed in triplicate.

2.6. Determination of the physicochemical properties of native and modified l-ASNase

2.6.1. Protease and sera treatment

Half ml (final concentration of 20 IU/ml) of native and modified l-ASNase were added to 2.5 ml phosphate buffer solution, pH 8.5 containing 50 IU trypsin. Samples were incubated at 37 °C for 30 min and residual enzyme activity of each sample was measured with 5 min interval. All experiments were performed in triplicate.

Half ml of each native and modified l-ASNase were loaded in 2.5 ml human serum at a final concentration of 20 IU/ml. After mild homogenization the samples were incubated at 37 °C for 70 h and in vitro half-lives of native and modified l-ASNase in human serum were detected by taking 0.5 ml of each sample with 10 h interval. All experiments were performed in triplicate.

2.6.2. Test of stability and activity in different pH and temperature

Determination of optimum temperature and thermal stability of native and modified l-ASNase was performed by incubation of enzymes at different temperature (25, 35, 45, 55, 65 and 70 °C) in 0.05 M Tris–HCl, pH 8.5 for 30 min with substrate asparagine at 100 mM. Sample with the highest activity was set as 100% of relative activity. All experiments were performed in triplicate.

Enzyme activity and stability of native and modified l-ASNase were estimated by incubation of each enzyme in different pH, from 3 to 12 by using 0.05 M acetate buffer (pH 3–5), 0.05 M Hepes (pH 6–8) and 0.05 M Tris–HCl (pH 9–12) as described in Section 2.5. All experiments were performed in triplicate.

2.6.3. Freezing–thawing

Five milliliter of native and modified l-ASNase (20 IU/ml) in 0.05 M phosphate buffer pH 8 were frozen at −20 °C slowly for 72 h. After each 12 h each sample was immersed in a water bath at 37 °C
and thawed for 15 min, then enzyme activity was measured and was freeze-dried again. All experiments were performed in triplicate.

2.6.4. Freeze–drying

Five ml of native and modified l-ASNase were frozen as described above and transferred to a freeze drier (ZirBus Technology GmbH, Germany) with condenser temperature $-80\,^\circ\text{C}$, chamber pressure (0.1 mbar) for 18 h. After this time the samples were withdrawn and reconstituted with 5 ml of 0.05 M phosphate buffer, pH 8 and enzyme activity were determined. This procedure was repeated for six time. All experiments were performed in triplicate.

2.6.5. ELISA to evaluate immunogenicity of native and modified l-ASNase

Nine adult male New Zealand White rabbits (about 1.5 kg body weight) were divided into three groups (three rabbits each groups). Native l-ASNase (with dose of 500 IU/kg), modified l-ASNase (with dose of 500 IU/kg) and inulin (5 mg/ml, 0.5 ml) were prepared in PBS and filtered by a 0.22 μm filter (Millipore, USA) to obtain sterilized samples. The animals in each groups were given intravenously (tail vein) injection on day 0, 4, 8, 12 and 15, respectively. On days 15 and 21 blood samples were aseptically collected by heart puncture and serum were isolated. Normal rabbit plasma (NRP) samples were obtained from rabbits at the experimental animal center at Shiraz Medical Sciences University, who had never received l-ASNase and used as negative control.

For the quantification of serum antibody (IgG) titer, 96 wells microtiter plates were coated with 100 μl of native and modified l-ASNase (5 μg/ml diluted in 0.05 M Tris–HCl, pH 8.5) and incubated over night at 4 °C. The plates were washed three times with water containing 0.4% Tween 20. The wells were blocked with 200 μl/well of 0.1% BSA and 0.05% Tween 20 in 0.1 M phosphate buffered saline (PBS) for 90 min. The plates were then washed three time with 0.05% Tween 20 in saline. For each l-ASNase preparation 100 μl of 1:1600 of plasma sample or NRP serum were added and incubated for 30 min at room temperature. After incubation, the plates were again washed three times as described above. A polyclonal goat anti rabbit IgG horseradish peroxidase conjugate was diluted to 1:8000 and 100 μl of this dilution was added to each well and incubated for 1 h at room temperature. The wells were drained and washed with Tween 20–saline three times and incubated with 100 μl of substrate solution containing 20 mg of o-phenylenediamine dihydrochloride in 50 ml of 0.1 M citrate buffer pH 6 and 150 μl of 3% H₂O₂ for 30 min at 37 °C in the dark. The reaction was stopped by addition of 100 μl, 0.1 M phosphoric acid solution. Optical densities were read spectrophotometrically at 450 nm by using an ELISA microplate reader (Awareness Technology Inc. USA). Assay was performed in triplicate.

3. Results and discussion

3.1. Modification and purification of oxidized inulin conjugated ASNase

In this study l-ASNase was modified with oxidized inulin polymer. This method has been used for the modification of E.coli and Erwinia l-ASNase by using oxidized dextran (Karsakevich et al., 1986; Wileman et al., 1983). The modified enzyme was prepared by the direct reaction of dialdehyde groups of oxidized inulin with e-amino groups of lysine and N-terminal amino groups of the enzyme after oxidation with sodium periodate.

Fig. 1 shows the results of G100 gel filtration chromatography of modified l-ASNase at molar ratios of 4:1 and 2:1 and native l-ASNase (Curves A, B and C, respectively). Modified l-ASNases at molar ratios of 4:1 and 2:1 that had higher molecular weight (curve A and B) were eluted from the column at first respectively, whereas the native l-ASNase with lower molecular weight was eluted in next fractions (curve C).

The formation of modified l-ASNase was confirmed by SDS-PAGE. As seen from electrophoresis analysis (Fig. 2), the modified l-ASNase of lower molecular weight was eluted in next fractions (curves B and C).
l-ASNases had broad high molecular weight bands with a wide range of molecular weight distribution (100–250 kDa). The migration rates of modified l-ASNase molecules (Lanes 3 and 4) were much slower than that of native l-ASNase (Lane 2). This would indicate that the glycation of l-ASNase resulted in greatly decreased electrophoretic mobility of the enzyme due to higher molecular weight. In lane 4, sharp bands were not seen because of the variation in the number of oxidized inulin molecules attached to the polypeptide chain of the enzyme. These multiple forms may also originate from molecules with different numbers of polysaccharides attached to each molecule of l-ASNase, extensive variation in conformation of different molecules, protein–protein interaction, isopeptide bond formation, or other unknown mechanisms (Diftis and Kiosseoglou, 2003). As shown in Fig. 2 the molecular weight of pure enzyme was about 33 kDa (Lane 2), corresponding to the subunit of l-asparaginase (Marlborough et al., 1975).

Based on the electrophoresis analysis, the extent of the conjugation of l-ASNase was increased progressively with increase in molar ratio of oxidized inulin to enzyme (Fig. 2 and Table 1). This result indicates the participation of more reactive aldehyde groups at molar ratio of 4:1 than 2:1.

Attachment of oxidized inulin to the enzyme often takes place on the ε-amino group of lysine residues or at the N-terminus. There are 22 lysine residues in the primary structure of one subunit of l-ASNase isolated from E. coli and active site characterization of l-ASNase did not reveal the presence of lysine residues (Saito et al., 1976). Our results, based on the calculation of the free amino groups of the enzyme, indicate modification of 14 lysine residues (62%) when a molar ratio of 4:1 was used whereas under optimum condition of molar ratio of 2:1, six lysine residue (27%) was modified (Table 1). The conjugation was carried out at pH 9 and at this pH the proportion of the unprotonated amino groups is sufficiently high to allow reaction of these groups with the aldehyde groups of oxidized inulin. Based on the wide range of molecular weight of oxidized inulin that was used in this study detection of accurate mol of inulin molecules that attached to the enzyme was difficult.

The best results for l-ASNase immobilization on dextran and levan were obtained with 15–20% modification of the amino groups (Karsakevich et al., 1986; Vina et al., 2001). Therefore, we performed subsequent investigation by using modified l-ASNase at molar ratio of 2:1 with 27% modification of amino groups [with residual activity about 65%]. On the other hand by using higher molar ratio of oxidized inulin/ enzyme (4:1), an increase in degree of modification (62%) but a decrease in residual activity (33%) was achieved. This condition is probably due to the participation of more reactive aldehyde groups at higher concentration of oxidized inulin and to increasing steric hindrance by conjugated branched inulins that might interfere with enzyme and substrate reaction (Vina et al., 2001). Other investigators have reported similar results by using different molecules binding to l-ASNase including dextran sulfate (Karsakevich et al., 1986), chitosan (Qian et al., 1996), polyethylene glycol (Soares et al., 2002), levan (Vina et al., 2001), silk sericin (Zhang et al., 2004) and silk fibroin (Zhang et al., 2005).

The formation of multiple forms of enzyme after its conjugation with inulin resulted in non efficient purification of modified enzyme by gel filtration chromatography and broadening their molecular weights. Although these multiple forms of enzyme had the same immunological and physicochemical properties, such stabilized bioconjugates may make problems for medical purposes. Nonetheless our studies on a bioconjugated form of l-ASNase that is commercially available (PEG-ASNase or pegaspargase) (Rizzari et al., 2006) showed that PEG-ASNase had the same molecular weight features based on the electrophoresis analysis (data not shown). To dissolve this problem (minimize the molecular weight broadening) we are attempting to establish different conditions such as different methods of chromatography, pH and temperature to produce the highest yield of pure inulin conjugated enzyme in our laboratory for in vivo study of cancer therapy.

### 3.2. Physicochemical and enzyme kinetic properties

The Lineweaver–Burk plots with relative rate (V⁻¹) versus substrate concentration (S⁻¹) for native and modified l-ASNase are shown in Fig. 3. Tow corresponding linear equations for native and modified l-ASNase were obtained from plots, $y = 10.739x + 1.578$ $(R^2 = 0.999)$ and $y = 6.283x + 6.209$ $(R^2 = 0.994)$, respectively.

The values of the $K_m$ of native and modified enzyme were $0.57 \times 10^{-4} \text{M}$ and $0.1 \times 10^{-4} \text{M}$, respectively. It is shown that the conjugation of oxidized inulin to l-ASNase resulted in considerably increasing affinity between modified l-ASNase and its substrate asparagines about five time.

Results from stability against trypsin digestion are illustrated in Fig. 4. The results showed the higher stability of the modified l-ASNase than that of native enzyme against trypsin digestion. The native enzyme exhibited a reduction of 90% of initial activity after 30 min whereas the modified l-ASNase retained 70% of enzyme activity after this time.

According to the results that are summarized in Fig. 5 the native l-ASNase had half life about 20 h whereas modification of l-ASNase with oxidized inulin resulted in extension of its circulating half life about 55 h.

Stability against protease digestion and increased half life of the enzyme depend on the presence of non specific antibodies or protease in the serum (Soares et al., 2002). Results from this

### Table 1

Degree of ε-amino group modification and enzyme activity of native and oxidized inulin conjugated l-ASNase at different ratio of oxidized inulin/ASNase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molar ratio of oxidized inulin/enzyme</th>
<th>ε-Amino group Modification (%)</th>
<th>Enzyme activity (%)</th>
<th>$K_m$ (M × 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>–</td>
<td>0</td>
<td>100</td>
<td>0.57</td>
</tr>
<tr>
<td>Modified A</td>
<td>2:1</td>
<td>27</td>
<td>65</td>
<td>0.1</td>
</tr>
<tr>
<td>Modified B</td>
<td>4:1</td>
<td>62</td>
<td>33</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Not determined.
The results of relative activity of native and oxidized inulin conjugated L-ASNase after trypsin digestion. Relative activity were measured on the basis of the highest activity of native and modified L-ASNase as 100%. All data were average value of triplicate measurement.

Fig. 4. The results of relative activity of native and oxidized inulin conjugated L-ASNase after trypsin digestion. Relative activity were measured on the basis of the highest activity of native and modified L-ASNase as 100%. All data were average value of triplicate measurement.

The study suggests that probably the modification with oxidized inulin protects L-ASNase by causing steric hindrance to the binding of antibodies or to the enzymatic hydrolysis caused by proteases and this modified enzyme can be considered as a drug with longer plasmatic half-life and improved therapeutic properties.

Fig. 5. The in vitro half-lives (arrows) of native and modified L-ASNase in human serum. Relative activity were measured on the basis of the highest activity of native and modified L-ASNase as 100%. All data were average value of triplicate measurement.

The optimum temperature for native L-ASNase was found to be about 55 °C and for modified enzyme was about 50 °C. Based on the remaining activity of 100% for native L-ASNase at different temperature, only 28% of the original activity remained at 65 °C after 30 min, however, the modified enzyme was more stable and after 30 min at 65 °C, it retained 66% of the initial activity. It is noteworthy that the optimum temperature range of the modified enzyme is wider than that of native L-ASNase that had maximal activity between 35 and 45 °C.

Improvement of thermostability of modified L-ASNase may be acquired by multipoint attachment of the polysaccharide chain to the enzyme molecule, resulting in greater rigidity of enzyme conformation and increasing of the activation energy for “unfolding” the enzyme (Shepard et al., 1983). It seems, therefore, reasonable to suggest that the stabilization of tetrameric L-asparaginase upon glycosylation may be due to protection from unfolding especially under extreme conditions. Marked improvement in thermostability of L-ASNase in this study was obtained in agreement with results of our studies on glycation of lysozyme by dextran (Amiri et al., 2008) and dextran, galactomannan and mannann (Scaman et al., 2006).

As shown in Fig. 7, the optimum pH range of modified L-ASNase was significantly broader than native enzyme and was between 5 and 8, but that of native enzyme was between 7.5 and 8. Modified enzyme was more stable in lower and higher pH in comparison with native enzyme (Fig. 7). The activity of the native enzyme decreased rapidly at pH below 7.5 and above 8, while modified enzyme activity was dropped below pH 5.5 and above pH 9. The possible mechanism of protein stabilization in a wide range of pH is protection of active sites by the inulin polymer chains. Without the protecting effect, the structure in the active site of the native enzyme is more susceptible to lower pH, leading to an irreversible change in the active site (Stechera et al., 1999). The enzyme activity is maximum at alkaline pH, probably due to produced L-aspartic acid acting as competitive inhibitor for enzyme in acidic condition (Miller et al., 1993) and due to protective effect of inulin polysaccharide chains against binding of L-aspartic acid, this inhibitory effect in low pH is decreased.

Based on the results that shown in Fig. 8 the modified enzyme retained 92% of its activity after repeated freezing and thawing for 72 h while native enzyme had only 70% of the initial activity after same period and condition.

3.3. Immunogenicity

The modification of L-ASNase with oxidized inulin at molar ratio of 2:1 resulted in decreasing antibody (IgG) titer and immuno-
or lowering enzyme activity by deposition of antigen–antibody complex in the reticuloendothelial system (Killander et al., 1976). The decreased antigenicity of the oxidized inulin modified l-ASNase can be thought as a consequence of masking the protein antigenic sites by the polymer. Moreover, a hindering effect due to the polymer cloud around the protein structure can, to some extent, avoid the Ag/Ab interactions. Fall in the immunogenicity might have led to production decrease in the neutralizing antibodies which can cause inactivation or inhibition of the enzyme.

4. Conclusion

The aim of this study was improvement of the physicochemical and decreasing the immunogenicity of anti-leukemic enzyme; l-asparaginase by conjugation to the oxidized inulin polymer. The modified enzyme increased the thermostability, in vitro half life, resistance to protease digestion, reusability after repeated freezing–thawing and widened the range of optimum pH. The $K_m$ of the modified enzyme was more than five times lower than that of the native l-ASNase, showing affinity between l-ASNase and its substrate has increased after bioconjugation. The immunogenicity of the modified l-ASNase was significantly decreased compared with the native enzyme. This method of l-asparaginase modification by using oxidized inulin indicates a promising stabilized product that may be employed for medical purposes in future.

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References


