ENDOPROLYLPEPTIDASE (EC 3.4.21.26), IMPROVEMENT OF THE ISOLATION METHOD

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The improvement of the enzyme isolation method consists in the addition of sodium sulphite (5 mmoles l^{-1}) used in the isolation procedure instead of the low-molecular thiols used previously, e.g. 2-mercaptoethanol, cystein, dithiothreitol *etc.* The protective effect against oxidation of the enzyme by aerial oxygen has been retained while, at the same time, autolysis of the enzyme was decreased and the final enzyme preparation exhibited higher values of specific activity.

Endoprolylpeptidase (EC 3.4.21.26) is a serine protease, the molecules of which contain in addition free thiol groups (2 to 3 groups per molecule) that are essential for maintaining the activity of the enzyme[1,2]. The enzyme is inactivated when these thiol groups are chemically modified. In order to protect these free thiol groups, an excess of low-molecular thiols is added to enzyme solutions in the course of isolation, as is conventionally also done with other enzymes, *e.g.* thiol proteases. For example, 2-mercaptoethanol[3], cystein[4], dithiothreitol[5] have been used in the isolation of endoprolylpeptidase. In the presence of low-molecular thiols the enzyme is effectively protected against oxidation, while it is also activated to such a degree, that it can undergo autolysis, which decreases the purity and activity of the final preparation.

This communication reports a simple method of overcoming the difficulties mentioned, caused by the use of low-molecular thiols as protective agents.

EXPERIMENTAL PART

Material and Methods

DEAE-Sephacel was purchased from Pharmacia (Uppsala, Sweden), DEAE-Cellulose DE32 was obtained from Whatman Ltd. (Mainstone, England). Fast Garnet GBC salt (diazotized O-aminoazotoluene) from Fluka AG (Buchs, Switzerland) and Triton X-100 from Serva (Heidelberg, Germany). Benzyloxycarbonylglycyl-proline 2-naphtylamide was synthesized as published[5].

Purification procedure: The enzyme was isolated by the method published previously[3] with the following difference: a buffer B of the following composition was employed in all isolation steps: Na-phosphate, pH 6·5, 20 mmoles l^{-1} ethylenediaminetetracetate 2 mmoles l^{-1} and sodium sulphite, 5 mmoles l^{-1}. All isolation steps were carried out at 4°C unless stated otherwise. Dialysis was carried out against buffer B, before final lyophilisation the enzyme preparation was dialyzed against a 2-mercaptoethanol solution, 1 mmole l^{-1}.

Enzyme assay. Enzyme activity was determined by measuring the rate of enzymatic hydrolysis of the synthetic substrate benzyloxycarbonylglycyl-proline 2-naphtylamide using a previously published method[5].

Protein content was measured by the Lowry and coworkers method[6].

Electrophoresis on polyacrylamide gel was done in discontinuous buffer systems according ot Davis[7] (pH 9·5) and according to Williams and Reisfeld[8] (pH 8·0).

RESULTS AND DISCUSSION

Table I summarizes the results of the isolation procedure described. For comparison, Table I includes results obtained with the original method of isolation in which 2-mercaptoethanol 2 mmoles l^{-1}, served as protective agent. It is to be seen from the comparison, that specific activity increased more than twice. Purity of the preparation was confirmed by analytical electrophoresis on polyacrylamide gel in discontinuous buffer systems at pH 9·5 and 8·0.

The use of sodium sulphite for protection of free thiol groups of the enzyme was motivated by the attempt at finding a mild reductant, capable to prevent efficiently the oxidation of thiol groups without causing undue activation of the enzyme (in order to prevent the rate of autolysis from rising too high particularly in later phases of purification). Sodium sulphite is conventionally employed as reducing and protective agent in solutions of hydroquinons and its derivatives.

TABLE I

Summary of endoprolylpeptidase isolation in the presence of sodium sulphite, 5 mmoles l^{-1}. Data in brackets are values of activity and degree of purification of the enzyme obtained by a similar procedure, using 2 mmoles l^{-1} 2-mercaptoethanol

Isolation step	Activity yield	Specific activity pcat/mg	Degree of purification
1. Extraction	100(100)	2·5(3·8)	1(1)
2. Acetone precipitation	100(100)	11·7(14·7)	4·7(3·8)
3. DEAE-Sephacel chromatography	95(121)[a]	1 500(713)	600(186)

[a] See reference[3].

We assume that in addition to the protective function, consisting in elimination of oxygen and other possible oxidants present in enzyme solutions, sodium sulphite can also react with the SH-groups of the enzyme, forming an enzyme S-sulphonate. The latter compound may be decomposed in the last step of the preparation procedure in the presence of 2-mercaptoethanol, generating the free active enzyme.

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