

PROCESS FOR THE CROSS-LINKING OF PROTEINS

The present invention relates to a process for cross-linking of proteins. More specifically the present invention relates to the novel use of new and known compounds for cross-linking of proteins and especially of enzymes.

Enzymes have been used extensively in industrial processes both in soluble and insoluble form. The use of soluble enzymes for industrial processes, however, is limited by their cost, their instability, and the difficulties in recovering them after the operation.

These disadvantages have been circumvented by a new technology based on enzyme immobilization, i.e. enzyme attachment to solid support materials. A number of techniques have been developed for enzyme immobilization, of which the major ones are intra molecular cross-linking and covalent linking to supports. Support materials in different forms - beads, membranes and fibers - can be used. The most common support material for enzyme reactors are beads or porous particles which can be packed into columns or used in stirred-tank reactors. Another form is the sheet or membrane form which can be used in pressure cells.

The advantage of utilizing such immobilized or insoluble enzymes resides in the possibility of acting catalytically with an enzyme on a substrate stream in a continuous way with no need of separating the enzyme from the product obtained by the catalytic reaction. Thus, the use of certain insoluble enzymes for the performance of enzymatic reactions is known and the following known methods of use can be mentioned, for example:

1. The insoluble enzyme particles are suspended in a tank with stirring, the out-going flux passing through a filter; and
2. The insoluble enzyme particles are packed into a column which is continuously flown through by the substrate.

More recently in our Israel Pat. specification No. 46 178 we have described and claimed a method for the performance of enzymatic reactions which comprises applying pressure to an aqueous solution of a substrate, which substrate may be chemically altered by way of an enzymatic reaction, causing thereby said solution to flow through a cross-linked enzyme membrane (as defined) and removing from the solution the product obtained by methods known per se.

An enzymatic membrane in connection with said process was defined as meaning a microporous membrane in the pores of which enzymes are cross-linked by way of a bifunctional coupling agent.

The present invention relates to the discovery of several new classes of multifunctional compounds which can serve as cross-linking agents for proteins and especially enzymes and which are utilizable in any context wherein cross-linked proteins and enzymes are required.

Cross-linking agents of proteins are bi- or multifunctional compounds capable of covalently binding with proteins to form either a long chain or a three dimensional polymeric structure as explained. Cross-linking agents are useful for enzyme immobilization in various modifications. Thus enzymes can be insolubilized by cross-linking them alone or in the presence of a co-

protein and usually enzymes are cross-linked within a water insoluble support.

The advantages of using multifunctional reagents as insolubilizing agents is that one reagent can be used to prepare different types of immobilized enzymes. The method is simple and non-specific.

The preparation of water insoluble enzyme-derivatives using multifunctional reagents, involves the covalent bond formation between molecules of the enzyme and the reagent to give intermolecularly cross-linked species.

Multi-functional reagents, i.e., cross-linking agents, can be used in several ways for the cross-linking of proteins, e.g.:

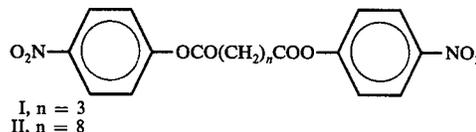
- (a) The pure enzyme or protein is reacted with the cross-linking agent to form three-dimensional species which are completely insoluble in water, or complex oligomeric soluble derivatives;
- (b) The enzyme is cross-linked in the presence of a second protein, to form a co-cross-linked derivative;
- (c) The enzyme is first adsorbed on an insoluble, surface-active support and then cross-linked intermolecularly with a multifunctional reagent; or
- (d) The enzyme can be cross-linked to a protein matrix.

Many cross-linking agents have been described and used in the past. Usually these are low molecular weight bifunctional reagents capable of binding with various sites of proteins. Some of them are soluble in water and usually they are very reactive and non-selective. Thus diisocyanates may react with amino, thiol and hydroxyl residues of proteins as well as with water. Glutaraldehyde which has been extensively used in the past is also nonselective. In some cases a reduced enzymatic activity may be explained by inactivation caused by the chemical alteration with the cross-linking agent. The following properties of a cross-linking agent for enzymes are required:

- (1) A complete insolubilization should be obtained.
- (2) The enzymatic activity should not be greatly reduced.
- (3) A chemically stable bond should be formed.

It is quite obvious that rather mild conditions during cross-linking are preferred, and extreme pH and temperature values as well as high concentration of organic solvents should not be used as these may cause a considerable inactivation of some enzymes.

A group of cross-linking agents which reacts selectively with amino groups consists of activated esters of di-carboxylic acids. Compounds such as di-p-nitro-phenyl glutarate (I) and di-p-nitrophenyl sebacate (II) are typical examples:



The main advantage of these cross-linking agents is their selectivity towards amine residues such as ϵ -amino lysine and α -amino acids. Modification of these amine residues usually does not greatly reduce enzymatic activity. The use of these cross-linking agents is practically limited for the following reasons: 1. These compounds are practically water insoluble and therefore