Hydroxyproline content of various highly purified acetylcholinesterases

| AcChE from | Specific activity (U/mg) | Mode of solubilization | Hydroxyproline |
|---|---|---|---|
| *Bungarus multicinctus* | 4290 | water soluble | 0.023 ± 0.02% |
| *Naja naja atra* | 5470 | water soluble | 0.024 ± 0.02% |
| Human erythrocytes | 4270 | 0.2% Triton X-100 | 0.037 ± 0.03% |
| Bovine erythrocytes | 4130 | 0.5% Triton X-100 | 0.043 ± 0.03% |
| Bovine nucleus caudatus | 4250 | 0.6–0.8% Triton X-100 | 0.035 ± 0.03% |
| *Electrophorus electricus*[1,7] (14S + 18S-form) | - | 1 M NaCl | 0.83 ± 0.13% |
| *Electrophorus electricus*[1,7] (11S-form) | - | proteolytic digestion | 0.1 ± 0.06% |

purified acetylcholinesterases from various other sources for hydroxyproline and hydroxylysine.

*Material and methods.* The acetylcholinesterases shown in the table (except those from electric fish) were solubilized by the method mentioned and purified to the indicated high specific activities by repeated affinity chromatography[2, 3]. Purity of the enzymes was demonstrated by electrophoresis under non-denaturing conditions, and by the fact that specific activity did not increase on further purification steps like affinity chromatography, electrophoretic[4] – or centrifugal[5] – separation of single enzyme forms. Hydroxyproline was analyzed by a modified method from Bondjers and Björkerud[6], hydroxylysine using an aminoacid analyser (Biocal BC 201).

*Results and discussion.* Hydroxylysine was absent in all acytylcholinesterases examined. The table shows that none of the enzymes contains hydroxyproline in such a significant amount similar to the 14S/18S-forms of electric fish acetylcholinesterase. We therefore suppose that the acetylcholinesterases examined do not possess a collagen-like 'tail', although they are extracted and purified under non-proteolytic conditions like the 14S/18S-forms of electric fish acetylcholinesterase. With exception of the 2 water-soluble 'snake' enzymes, the acetylcholinesterases are membraneous proteins. The way of anchoring acetylcholinesterase to the membrane, however, must be quite different from what is found in electric tissues. This assumption is supported by the fact that very different methods must be applied for solubilizing the acetylcholinesterases mentioned. Whereas the collagen-like 'tail' containing acetylcholinesterase from *Electrophorus electricus* can easily be solubilized with 1 M NaCl, high salt concentration severely inhibits solubilization of acetylcholinesterase from bovine nucleus caudatus. This enzyme slowly solubilizes to a large amount at very low salt concentration, whereas under the same conditions, solubilization of acetylcholinesterase from human erythrocytes does not take place and acetylcholinesterase from bovine erythrocytes is only dispersed in particles. A unifying concept of anchoring acetylcholinesterase to the membrane seems to be impossible.

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## The halophilic properties of pyruvate kinase from *Vibrio costicola*, a moderate halophile

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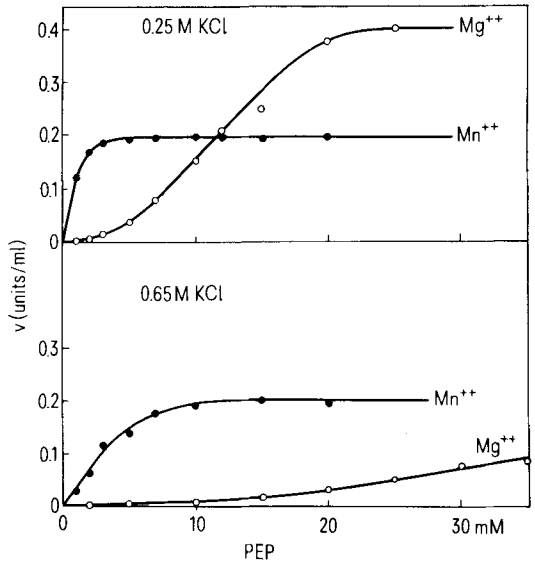
*Summary.* Pyruvate kinase from *Vibrio costicola*, a moderate halophile, appears to be adapted to functioning in the presence of salt. Its stability depends on the ionic strength of the medium. The amino acid composition resembles that of other halophilic enzymes. It is proposed that the halophilic pyruvate kinase utilizes preferentially the $Mn^{++}$ cofactor which forms more stable complexes in the presence of physiological concentrations of salt.

*Vibrio costicola* is a moderately halophilic bacterium having an intracellular salt concentration of 0.95 M $Na^+$ and 0.66 M $K^+$ [1]. The cytoplasmic pyruvate kinase purified from the bacterium appears to be inhibited by NaCl and KCl to an extent which is not compatible with the physiological conditions[2]. The stability of pyruvate kinase, however, is so dependent upon the presence of salt in the solvent that it is improbable that the enzyme is sequestered in a compartment at low ionic strength in vivo. The enzyme is stable in 30% (v/v)glycerol at room temperature but in solution devoid of glycerol, the enzyme is inactivated within a few days. The enzyme is less stable at low ionic strength and requires salt for increased stability. This characteristic is shared by other halophilic enzymes purified from extremely halophilic bacteria. It has been proposed that the amino-acid composition of halophilic proteins could be different from that of non-halophilic proteins, in order to explain the adaptation of the 3-dimensional structure to salt environments. The amino acid composition of pyruvate kinase from *Vibrio costicola* has been determined. Purified pyruvate kinase[2] was hydrolyzed in sealed tubes with 5.6 N HCl at 110 °C for periods of 24, 48 and 72 h. The hydrolysates were analyzed on a Technicon TSM using the type A resin. Amino-acids values were corrected with an internal standard of norleucine. The values of Asx, Arg and Ser were extrapolated to zero time and the value of Thr was that measured at 72 h of hydrolysis. A peak corresponding to ornithine was found and its content was checked by mass spectrometry. Table 1 shows the amino-acid composition of pyruvate kinase from *Vibrio costicola* and the amino-acid compositions of pyruvate kinases from other sources for comparison. Ornithine is a

rare amino-acid which is not coded for by DNA triplets. The presence of non-coded amino acids in proteins is usually attributed to post-translational modifications of enzymatic origin[7]. Ornithine has never been reported in halophilic proteins but is found in some proteins, including $\alpha_1$-$\alpha_2$ globulin[8] and a lectin from potato tubers[9]. A comparison of the amino-acid composition of pyruvate kinase from *Vibrio costicola* with pyruvate kinases from bacterial and mammalian sources, shows an increased proportion both of Glx residues[10] and of serine and glycine, as well as a decreased number of hydrophobic residues. Table 2 shows that these properties are usually shared by other halophilic proteins for which the amino-acid composition is known: an extracellular nuclease from *Micrococcus varians*[11], a high potential iron-sulfur protein from *Halococcus*[12], malate dehydrogenase from *Halobacterium* of the Dead Sea[13], a DNA-dependent RNA polymerase from *Halobacterium* of the Dead Sea[15]. The composition is always referred to that of the corresponding non-halophilic bacterial enzyme. It must be pointed out that, in the cases of the extracellular nuclease and the RNA polymerase, the molecular weights of the halophilic and non-halophilic proteins are quite different. Since globular proteins have polar and charged groups exposed to the solvent, and non-polar groups buried in the interior of the protein, globular proteins have a definite ratio of polar to non-polar amino-acids determined by the surface to volume ratio[16]. This ratio is increased at lower molecular weight. This may be the explanation for the apparent lower content of acidic residues in the halophilic RNA-polymerase. It has been proposed that the excess of acidic residues in halophilic proteins binds cations at high salt concentration and that denaturation of halophilic enzymes at low ionic strength might be due to the charge repulsion between unshielded acidic residues[17]. On the other hand, high ionic strength is known to reinforce hydrophobic bonds and it has been proposed that in halophilic proteins, hydrophobic residues could be replaced by mildly hydrophobic residues like glycine and serine in order to maintain the balance between hydrophobic and hydrophilic forces[18]. Since the optimal conditions for stability of pyruvate kinase from *Vibrio costicola* corresponded to the intracellular salt concentrations in the cell, it was decided to investigate the conditions in which the enzyme was reasonably active in the presence of salt. Pyruvate kinase is an enzyme which functions with a divalent cation, $Mg^{++}$ or $Mn^{++}$ as cofactor[2]. We wished to investigate whether $Mn^{++}$ was a better cofactor than $Mg^{++}$ at high salt concentration. The figure shows the rate dependence of pyruvate kinase on the substrate phosphoenol pyruvate concentration at 0.25 M and 0.65 M KCl. The extent of inhibition by KCl is lower with $Mn^{++}$ than with $Mg^{++}$. The hypothesis is presented that, in vivo, the enzyme may preferentially utilize $Mn^{++}$ as cofactor. Support for this theory is provided by the fact that other halophilic enzymes, such as the manganese-ion-stimulated alkaline phosphatase recently discovered in *Halobacterium cutirubrum*[19], have manganese as cofactor. It has been known for a long time that the manganese-ion complexes are more stable that the magnesium-ion complexes[20]. They




Rate dependence of pyruvate kinase from *Vibrio costicola* on phosphoenolpyruvate concentration at 0.25 and 0.65 M KCl, in the presence of $Mn^{++}$ or $Mg^{++}$. The assay is coupled to the lactate dehydrogenase reaction at room temperature and the disappearance of NADH is followed spectrophotometrically at 340 nm. 1 ml of assay solution contains: PIPES 75 mM, pH 6.7, $MnCl_2$ or $MgCl_2$ 11.2 mM, ADP 4 mM, LDH 100 units and NADH 0.2 mM.

Table 1. Pyruvate kinase composition in amino-acids (in mole %)

| | Human | | *E. coli* | | *V. costicola* |
|---|---|---|---|---|---|
| | M (3) | K (4) | I (5) | II (6) | |
| As (x) | 9.3 | 9.6 | 11.3 | 11.8 | 8.6 |
| Gl (x) | 11.1 | 10.0 | 10.2 | 8.0 | 16.0 |
| Lys | 6.8 | 6.4 | 7.3 | 6.1 | 3.9 |
| His | 2.4 | 2.4 | 1.7 | 1.5 | 3.4 |
| Arg | 5.6 | 5.9 | 4.1 | 6.3 | 3.4 |
| Orn | – | – | – | – | 4.2 |
| Gly | 7.7 | 5.7 | 8.9 | 9.2 | 15.3 |
| Ala | 10.2 | 9.5 | 9.2 | 11.3 | 8.4 |
| Val | 8.7 | 8.4 | 9.4 | 9.9 | 4.3 |
| Leu | 7.5 | 7.4 | 7.9 | 9.0 | 4.6 |
| Ile | 6.5 | 6.9 | 6.8 | 5.5 | 3.1 |
| Met | 3.6 | 5.5 | 3.4 | 3.2 | 0.9 |
| Phe | 3.3 | 3.3 | 2.6 | 1.9 | 2.0 |
| Pro | 4.1 | 5.1 | 2.8 | 2.9 | 2.5 |
| Ser | 5.6 | 6.3 | 5.6 | 5.9 | 14.0 |
| Thr | 5.2 | 5.5 | 7.3 | 6.3 | 3.5 |
| Trp | 0.6 | 0.6 | 0 | – | – |
| Tyr | 1.8 | 1.7 | 1.3 | 1.2 | 1.3 |

Table 2. Comparison of the amino-acid composition of halophilic and non-halophilic enzymes

| Enzymes | I | II | III | IV |
|---|---|---|---|---|
| AMP-activated pyruvate kinase | 240,000/190,000 | 3.8 | 17.9 | 14.2 |
| Extracellular nuclease | 99,000/17,000 | 24.8 | 0 | 5.9 |
| High potential iron-sulfur protein | 9,000/9,000 | 31.0 | 5.4 | (−5.1) |
| Malate dehydrogenase | 78,000/60,000 | 9.2 | 8.3 | 0 |
| DNA-Dependent RNA polymerase | ($\alpha+\beta$) 36,000/400,000 | (−6.9) | 17.5 | 18.6 |
| Glutamate dehydrogenase | 213,000/480,000 | 13.4 | 1.8 | 1.4 |

I Molecular weights $MW_h/MW_{nh}$; II Excess of acidic residues $(A\text{-}B)_h$-$(A\text{-}B)_{nh}$ (%); III Deficit of hydrophobic residues ($\psi_{nh}$-$\psi_h$) (%); IV Excess of serine and glycine $(Ser+Gly)_h$-$(Ser+Gly)_{nh}$ (%).

are thus able to compete more successfully with the potassium-ion complexes occurring in a KCl-rich medium.
We have thus demonstrated that pyruvate kinase from *Vibrio costicola* may function as a halophilic enzyme. Its halophilic character requires further testing in vivo.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## Silymarin, an inhibitor of lipoxygenase


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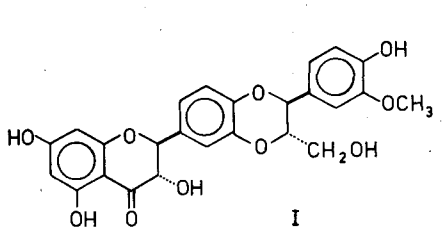
*Summary.* Silybin (**I**), silydianin (**II**) and silychristin (**III**), the main constituents of silymarin, non-competitively inhibit the lipoxygenase from soybeans (EC 1.13.11.12) in vitro.


Normal lipoid peroxidation is a physiological process which assists the natural decomposition and conversion of membrane lipoids (ML). On the other hand, pathologically increased lipoid peroxidation is regarded as an indication of lasting membrane damage, as under certain circumstances it can have deleterious consequences for the organs involved[3]. The integrity of animal and vegetable membranes is determined by the functional state of the ML's, or of the fatty acids (FA) in them, in particular polyunsaturated fatty acids. The biological properties of membranes, and their functions, such as permeability, active transport, inhibition of contact, conduction in nerves, enzymatic reactions, cellular immune reactions, etc. depend on the presence of ML and on the condition of the FA in the latter[4]. Furthermore, individual FA are precursors of prostaglandins[5].
The natural decomposition of ML is initiated by an attack of oxygen-carrying enzymes on the FA, which are released from the ML by lipolysis. Radical peroxides occur as primary reaction products, and can become the starting point for further oxidative changes in the ML and other cellular components. In an intact and healthy organism, physiological antioxidants and repair mechanisms ensure that these processes are kept within limits[5,6].
Certain toxins (producers of radicals) initiate an autocatalytic peroxidation similar to enzymatic lipoid peroxidation. The effects of this autocatalytic peroxidation exceed by far those of enzymatic lipoid peroxidation, and subsequently cause a rapidly progressive destruction of the pericellular and intracellular membranes[7]. The integrity of the ML is destroyed, and the result is the breaking down of the physiological membrane functions. Externally there are symptoms of poisoning, which, if the natural capacities for compensation and regeneration are overstretched by the excessive or continuous supply of the noxious agent, end in the rapid death of the individual or result in protracted invalidism[3].

The above-mentioned pathological lipoid peroxidation can be counteracted, in the case of experimental animals, for example, by silymarin, the 'antihepatotoxic principle'[8] of the fruit of the milk thistle[9,10-12]. For this reason silymarin, a complex consisting essentially of the active components silybin (**I**), silydianin (**II**) and silychristin (**III**)[13], is used therapeutically for various liver diseases[14].

For a long time the way in which silymarin works has been linked with a partial or total inhibition of the pathological changes in the ML described above[14]. However, the molecular function of substances **I-III** has not yet been explained completely. In the present investigation we looked into the question of whether, and to what extent, **I-III** influence the activity of lipoxygenase as specific effectors, and whether the effect of silymarin could possibly be due to an inhibition of enzymatic lipoid peroxidation.

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) catalyses the transfer of molecular oxygen to polyunsaturated FA, for example linolic acid, linoleic acid and arachidonic acid, the corresponding FA peroxides being formed in the process[5,6,16]. This enzyme is rich in iron[16] and is ubiquitous in the plant and animal king-



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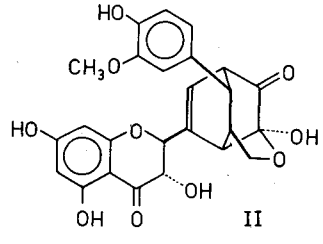
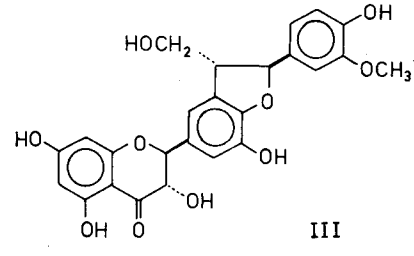


II



III