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Effect of pH and salts on the binding of free amino acids to the corn protein zein studied by thin-layer chromatography

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Summary. The interaction of free amino acids with the corn protein zein was studied by thin-layer chromatography carried out on cellulose layers covered with zein and the effect of pH and salts on the strength of interaction was elucidated. Only the binding of Arg, His, Lys, Orn and Trp to zein was verified, other amino acids were not retained. Retention of Arg, His, Lys and Orn decreased linearly with increasing concentration of salts the mobile phase indicating the hydrophilic character of amino acid–zein interaction. Both alkaline and acidic pH influenced the strength of binding. Principal component analysis indicated the different character of the influence of pH and salts on the interaction. The results suggest that these amino acid residues may account for the binding of other peptides and proteins to zein.

Keywords: Amino acids – Corn protein zein – Salt effect

Introduction

Interactions between amino acids, peptides and proteins have a paramount importance in many biochemical, biological and biotechnological processes. They influence protein structure (Spadaccini et al., 2001), modify biological efficacy (Chen et al., 2000), and enzyme activity (Fulop et al., 2001), participate in ligand recognition (Cordier et al., 2000), modulate the efficacy of active sites of proteinase (Strisovsky et al., 2000), etc.

The character of such type of interactions has been vigorously discussed the conclusions highly depending on the chemical structure of the interacting molecules. Thus, the involvement of electrostatic forces (hydrogen bonds) in the interaction of amino acids (Cox, 2000), in the determination of multiple turns in proteins (Guruprasad et al., 2000), in the modification of protein (Arnold and Oldfield, 2000) and peptide structure (Kasim and Swenson, 2000), in the aggregate formation of peptides (Mong et al., 2001), in the protein–protein interaction (Tanahashi and Tabira, 2000; Matern et al., 2000) and in the association of proteins (Wang et al., 2000) has been demonstrated. Furthermore, the interaction between the aromatic ring of phenyalanine side chain has been observed (Gorbitz, 2000). The simultaneous occurrence of hydrophobic binding forces and hydrogen bonds has also been reported (De Beer et al., 2000; Wu et al., 2000). It has been illustrated that both hydrophobic and electrostatic interactions may influence peptide-protein binding (Liu et al., 2000) and protein–protein associations (Nusrat et al., 2000).

Chromatographic techniques have been frequently employed for the assessment of various molecular interaction of biological relevance (Cserháti and Valkó, 1994). The use of thin-layer chromatography (TLC) for the study of such interactions has marked advantages: the method is generally rapid, it is easy to carry out, makes possible the simultaneous determination of numerous interaction on one plate, and the amount of the more hydrophobic interactive compounds is extremely low (Zarzycki et al., 1999).

Principal component analysis (PCA), a versatile and easy-to-use multivariate mathematical-statistical method has been developed to contribute to the extraction of maximal information from large data matrices containing numerous columns and rows (Mardia et al., 1979). PCA allows the elucidation of the relationship between the columns and rows of any data matrix without being one the dependent variable. PCA is a so-called projection method representing the original data in smaller dimensions. It calculates the correlations (similarities and dissimilarities) between the columns of the data matrix and

classifies the variables according to the coefficients of correlations taking into considerations simultaneously the magnitude and sign of the coefficients of correlation. PCA has been frequently employed in many fields of up to date research. Thus, it has been used in quantitative structure-activity relationship (QSAR) studies (Drew et al., 1998), for the exploration of molecular structure-property relationships (Seybold, 1999), for the evaluation of molecular lipophilicity (Sarbu and Todor, 1998; Mannhold et al., 1998), for theoretical organic chemistry (Héberger and Lopata, 1998), for quantitative structure-retention studies in chromatography (Héberger and Görgényi, 1999), for the elucidation of structure-biodegradation relationships (Damborsky et al., 1998), for the clustering of amino acids (Zaliani and Gancia, 1999), for the assessment of solvent properties (Katritzky et al., 1999), and polarity indicators in gas-chromatography (Héberger, 1999), etc.

The objectives of the study were the determination of the interaction of free amino acids with the corn protein zein, the elucidation of the influence of pH and various salts on the interaction and the evaluation of the data by PCA.

Materials and methods

L-Amino acids of analytical purity have been purchased from REANAL FINE CHEMICALS (Budapest, Hungary) and were used as received. Microcrystalline cellulose for thin-layer chromatography and solvents of HPLC quality have been obtained from Merck (Darmstadt, Germany). Zein-coated cellulose stationary phase has been prepared by dissolving 0.5 g zein in the mixture of 160 ml of *n*-propanol and 40 ml of water at 70°C under continuous gentle stirring. After the dissolution of the protein 20 g of microcrystalline cellulose was added and the mixture was stirred for two hours at the same temperature. Solvents have been removed at 70°C in vacuum. Plates of 20×20 cm containing 5 g of stationary phase have been prepared. Untreated cellulose plates served as controls. Amino acids have been dissolved in distilled water: 2-propanol at the concentration of 1 mg/ml, and 2 μl of the solutions were spotted onto the plates. Amino acids have been developed in distilled water as mobile phase and in aqueous solutions of acetic acid, sodium acetate, sodium chloride and magnesium chloride the concentration of additives varying between 150 and 0.5 mM. Plates have been developed in sandwich chambers ($22 \times 22 \times 3$ cm) at ambient temperature; the development distance was approximately 16 cm. After development the plates have been dried at 105°C and the amino acids have been detected by the ninhydrin reagent. Each experiment has been run in quadruplicate. The R_M values characterizing the retention of solutes in reversed-phase thin-layer chromatography (RP-TLC) have been calculated by

$$R_M = \log(1/R_f - 1) \quad (1)$$

for each amino acid in each mobile phase. When the coefficient of variation of the parallel determinations was higher than 6% the R_M value was omitted from the subsequent calculations. In order to elucidate the effect of eluent additives on the binding of amino acids to zein and cellulose the following equation has been fitted to the experimental data:

$$R_M = R_{M0} + b \cdot C \quad (2)$$

where R_M is the R_M value of an amino acid determined in a given mobile phase; R_{M0} is the R_M value determined in distilled water; b is the change of the R_M value caused by unit change of the concentration of the eluent additive (related to the sensitivity of the strength of the binding of amino acids to zein towards eluent additives), and C is the concentration of additive. Calculations have been performed only for Arg, His, Lys, Orn and Trp because other amino acid did not bind to zein. Calculations have been carried out separately for cellulose and impregnated cellulose stationary phases and for the four eluent additives.

The similarities and differences among the amino acids and eluent additives has been assessed by PCA. The four eluent additives were the variables and the b values of Eq. (2) for Arg, His, Lys, and Orn determined on cellulose and on impregnated cellulose layers were the observations (altogether 8 variables). Trp has been omitted from PCA because its R_M value did not depend significantly on the concentration of additives in the mobile phase. The variance explained was set to 99%.

Software for PCA has been prepared by Dr. Barna Bordás, Plant Protection Institute of Hungarian Academy of Sciences (Budapest, Hungary).

Results and discussion

The majority of amino acids was not retained neither on cellulose not on zein-coated cellulose layers that means that they do not bind to these stationary phases. Only amino acids Arg, His, Lys, Orn and Trp showed measurable retention, their mobility was slightly different on cellulose and on zein-coated cellulose layers suggesting the amino acids really interact with the molecules of the protein. The retention of each amino acid except Trp decreased monotonously with increasing concentration of the additive in the mobile phase indicating that the strength of interaction of amino acids with zeinis reduced in ionic environment. This result can be tentatively explained by the supposition that the amino acid–zein interaction is of polar character and amino acids are bound either to the polar peptide bonds or to the polar side chains of the protein. The R_M value of Trp did not depend significantly on the concentration of additives in the mobile phase. This finding can be tentatively explained by the supposition that the hydrophobic part of Trp can bind with hydrophobic forces to the apolar substructures of zein while the polar head group interacts with the same molecular substructures than the other amino acids. As the eluent additives decrease the strength of hydrophilic interactions and increase the strength of hydrophobic ones the unusual retention behaviour of Trp is the result of the interplay of both types of interactive forces. The parameters of the linear relationships between the R_M values of amino acids and the concentration of additives in the mobile phase are compiled in Table 1. The relationship was significant at a significance level of 95% in each instance demonstrating the applicability of Eq. (2). The coefficients of correlation were between 0.8481 and 0.9868 confirming the good reproducibility of the RP-TLC system. Both parameters of Eq. (2) (R_{M0} and b) were different for cellulose and zein-coated cellulose stationary phases proving again the

Table 1. Parameters of the linear relationships between the R_M values of amino acids and the concentration of additives in the mobile phase (C). Cell = cellulose stationary phase; Zein = zein-coated cellulose stationary phase

$(R_M = R_{M0} + b \cdot C)$						
Amino acid	Stationary phase	Additive	Parameters			
			R_{M0}	$-b \times 10$	$s_b \times 10^{-2}$	$r_{calc.}$
Arg	Zein	Sodium chloride	1.03	2.76	5.23	0.8939
	Cell		0.51	6.13	8.69	0.9621
His	Zein		0.33	1.15	2.70	0.8481
	Cell		0.13	2.62	3.41	0.9677
Lys	Zein		0.31	1.56	1.91	0.9513
	Cell		0.56	7.91	12.10	0.9562
Orn	Zein		0.28	1.48	1.73	0.9550
	Cell		0.56	7.32	14.41	0.9304
Arg	Zein	Acetic acid	1.05	3.20	3.98	0.9566
	Cell		0.69	7.81	19.25	0.8969
His	Zein		0.33	1.24	2.66	0.8689
	Cell		0.14	3.38	4.86	0.9609
Lys	Zein		0.26	1.20	2.31	0.8911
	Cell		0.69	8.51	16.29	0.9341
Orn	Zein		0.33	1.26	2.05	0.9183
	Cell		0.65	7.55	14.29	0.9353
Arg	Zein	Magnesium chloride	0.84	3.90	7.38	0.9072
	Cell		0.52	9.87	25.98	0.9099
His	Zein		0.38	2.35	2.96	0.9555
	Cell		0.14	5.38	9.25	0.9584
Lys	Zein		0.26	2.49	2.22	0.9770
	Cell		0.41	10.47	26.22	0.9174
Orn	Zein		0.28	2.51	1.68	0.9868
	Cell		0.41	10.25	24.98	0.9212
Arg	Zein	Sodium acetate	0.88	2.36	5.23	0.8622
	Cell		0.61	7.81	16.43	0.9396
His	Zein		0.32	1.22	2.21	0.9031
	Cell		0.11	4.07	6.06	0.9682
Lys	Zein		0.23	1.30	2.98	0.8553
	Cell		0.76	9.80	33.74	0.8590
Orn	Zein		0.25	1.08	2.51	0.8685
	Cell		0.74	9.97	33.54	0.8640

Table 2. Similarities and differences between the binding characteristics of amino acids to the corn protein zein

Results of principal component analysis			
Number of principal component	Eigenvalues	Variance explained %	Total variance explained %
1	3.89	97.38	97.38
2	0.08	2.06	99.44

Results of principal component loadings		
Variable	Number of principal component	
	1	2
b_{NaCl}	0.99	−0.08
$b_{Acetic\ acid}$	0.99	0.00
b_{MgCl_2}	0.97	0.23
$b_{Naacetate}$	0.99	−0.15

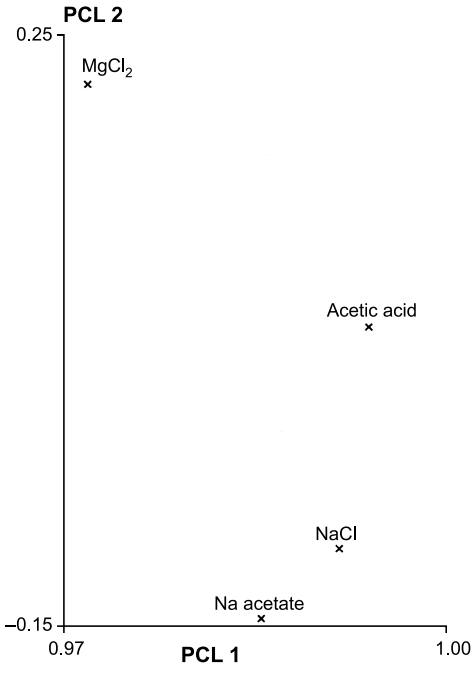

Fig. 1. Similarities and dissimilarities among the effects of eluent additives. Map of principal component loadings

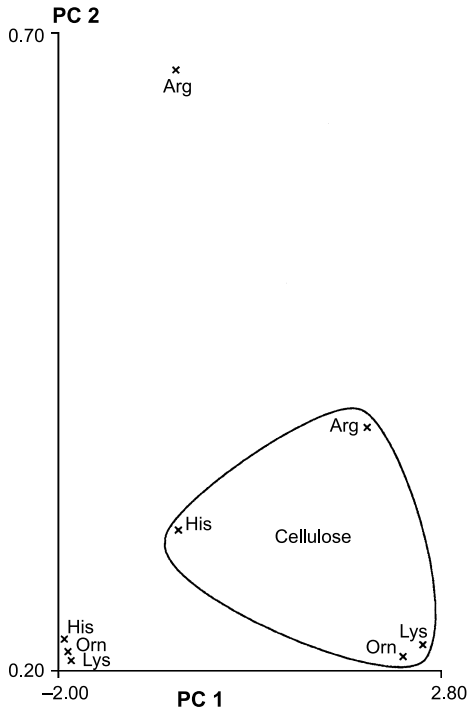

Fig. 2. Similarities and dissimilarities among the binding of amino acids to cellulose and zein-coated cellulose stationary phases. Map of principal component variables

occurrence of the binding of amino acids to zein. The results of PCA are compiled in Table 2. Two principal components explained 99.44% of the total variance that means that two theoretical (background) variables are sufficient to describe the effect of four eluent additives on the strength of amino acid–zein interaction. Unfortunately, PCA does not define these theoretical variables as concrete physical or physicochemical entities, only indicates their mathematical possibility. Each additive has a high loading in the first PC indicating the basic similarity of their influence on the retention of amino acids.

The map of PC loadings is shown in Fig. 1. Eluent additives are widely distributed on the map indicating that each of them exerts a separate effect on the amino-acid–zein binding, suggesting that both the pH and the charge of cation influence differently the binding of amino-acids to zein. This result entirely supports our previous conclusions that the binding of free amino acids to zein is mainly of hydrophilic character involving electrostatical interactions.

The map of PC variables are shown in Fig. 2. The distribution of amino acids on the map demonstrates the marked discrepancy between the retention characteristics of cellulose and zein-coated cellulose. Furthermore, the map illustrates that the effect of eluent additives on the binding of free amino acids is similar on cellulose stationary phase but considerably different on zein coated one demonstrating again the deviation between the binding characteristics of cellulose and zein-coated cellulose.

It can be concluded from the data that TLC carried out on cellulose and zein-coated cellulose stationary phases can be successfully employed for the determination of the interaction between free amino acids and the corn protein zein and can be used for the study of the effect of pH and the concentration of ions on the strength of interaction.

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