

# The effect of clay structure on peptide bond formation catalysis

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Received 8 May 1998; accepted 25 September 1998

## Abstract

The catalytic efficiency in peptide bond formation of various clays was tested by reactions of glycine (gly), diglycine (gly<sub>2</sub>), glycine + alanine (ala), gly<sub>2</sub> + ala. The main effects of clay structure and composition resulted as following: (1) Clay composition influences the activation of reactant molecules at clay particle edges. (2) Acidity (basicity) of the clay surface can change on the state of the reactant. (3) The clay structure is related to suspension stability and thus accessibility of clay catalytic sites. Mg-rich trioctahedral clays hectorite (smectite) and talc are the most efficient catalysts. Oligomerization of gly and gly<sub>2</sub> proceeds on all clays, whereas oligopeptides including ala units are formed with much lower yields and only on the most efficient catalysts. Besides dipeptides and other linear peptides, also cyclic anhydride (diketopiperazine) is formed with relatively high yields from diglycine. Cyclic anhydrides can directly act in the formation of the linear oligopeptides by ring opening molecular rearrangement and addition of another amino acid (oligopeptide). © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Amino acid; Catalysis; Clay; Prebiotic peptide bond formation

## 1. Introduction

### 1.1. Clay structure

Studies on the structure of the clay minerals have been summarized in several monographs [1,2]. Clay minerals are phyllosilicates, mostly composed of small or microscopic layered particles, and the layers consist of linked sheets of tetrahedra and octahedra. The tetrahedral sheets are formed by SiO<sub>4</sub><sup>4-</sup> tetrahedra, where Si atoms

are partially substituted by trivalent atoms (Al, Fe). Each of three ‘basal’ oxygen atoms are shared by two connected tetrahedra, the fourth one is linked to the octahedral sheet. Octahedra (MO<sub>4</sub>(OH)<sub>2</sub>, M = Al, Mg, Fe) are connected laterally by sharing octahedral edges. There are two types of octahedral sheets: (1) Al-octahedral sheet is called dioctahedral sheet and its structure is similar to that of  $\gamma$ -Al(OH)<sub>3</sub>. Al atoms occupy only two of three possible central positions of the octahedra. (2) Trioctahedral sheets, of brucite (Mg(OH)<sub>2</sub>)-like structure, contain mostly Mg central atoms. Clay layers can be formed from one tetrahedral sheet linked

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to one octahedral sheet—1:1 layer type (kaolinite; serpentine) or one octahedral sheet between two tetrahedral sheets—2:1 layer type (smectites, micas, vermiculite, chlorites, Fig. 1). According to the composition of the octahedral sheet there are di- or trioctahedral smectites, micas, chlorites, etc. Fibrous clays, attapulgite and sepiolite are a special case from the structural point of view. They are composed from layer bands linked in opposite direction by the tetrahedral oxygen atoms forming ‘inverted layers’. The surface of fibrous clays is characterized by the channels parallel to the chains.

Unequivalent isomorphous substitutions (e.g., Al(III) for Si(IV), Mg(II) for Al(III)) cause net negative layer charges, which are compensated in the interlayer spaces by hydrated cations (smectites, vermiculites), potassium cations (mica), or positively charged  $\text{Mg}(\text{OH})_2$  layer (chlorite). The layer charge of some clays is very small, approaching almost zero (kaolinite, pyrophyllite, talc).

### 1.2. Catalysis of amino acid condensation

Clays are known as minerals with particular suitability for adsorption and catalytic processes. They were probably present on the primitive earth crust after the formation of an early hydrosphere as the weathering products of volcanic rocks [3]. Clay minerals could, therefore, have played a crucial role in the molecular evolution of bio-organic compounds (amino

acids, peptides, sugars, nucleosides, nucleotides, nucleic acids) in chemical evolution [3–7]. The catalytic activity of some clays to promote oligomerization of the precursors of peptides [8–14] and nucleic acids [15,16] was confirmed in numerous experiments. Recent papers [17–22] showed, that clay catalysed peptide formation is a rather complex combination of various reactions (amino acid dimerization, cyclic anhydride formation and peptide chain elongation) different in their mechanism and dependence on reaction conditions. The majority of published data refers to experiments using model reactions of the simplest amino acid gly catalysed by montmorillonite or kaolinite [8,9,17–22]. Studies on smectite catalysed glycine and gly<sub>2</sub> oligomerization showed, how the catalytic efficiency depends on the smectite composition, the layer charge location and the type of exchangeable cations [17,18]. The objective of this work was to investigate experimentally the catalytic efficiency of various clays of different structure.

## 2. Experimental

### 2.1. Materials

Pyrophyllite and talc were obtained from the collection of the Department of Hydrosilicates (Institute of Inorganic Chemistry, Slovak Academy of Sciences, Bratislava). Montmorillonite SAz-2, hectorite SHCa-1, kaolinite KGa-1, vermiculite VTx-1, chlorite (corrensite) CorWa-1, sepiolite SepNev-1 and attapulgite PFI-1 were purchased from the Source Clays Depository of the Clay Mineral Society. Clays were ground and purified by sedimentation of water dispersions, according to the procedures given by the Source Clay Depository. The 2- $\mu\text{m}$  fractions were  $\text{Ca}^{2+}$  saturated, washed free of excess ions, dried at 50°C, ground to pass 0.2 mm sieve and used as catalysts.

Gly, ala and their oligopeptides of analytic grade purity were obtained from Sigma Chemical and used without further purification. Ala

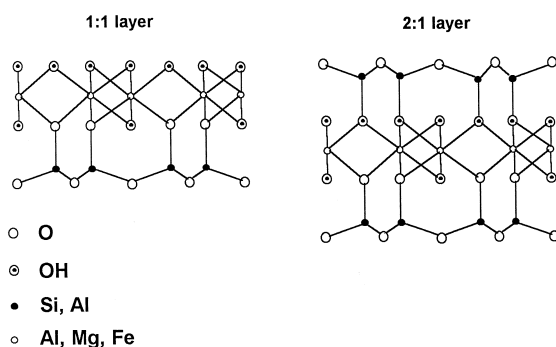


Fig. 1. 1:1 Layer and 2:1 layer type formed by linked tetrahedral and octahedral sheets.

and oligopeptides containing ala unit(s) were L-isomers.

## 2.2. Reaction system

Four reactions were carried out for testing the catalytic efficiency of clays, starting with gly, gly<sub>2</sub>, gly + ala, and gly<sub>2</sub> + ala, respectively. 0.01 g clay was mixed with 1 ml 10 mM reactant solution in 2-ml glass vials. In case of reactions with two reactants the concentration of both was 5 mM. The vials with suspensions were then heated in a heating box at 80 ± 1°C to complete evaporation within 24 h (one cycle). The residue was then taken up with 1 ml distilled water and subjected to the next cycle, until five cycles were completed. Five parallel experiments were carried out for each system. After the fifth cycle 1 ml CaCl<sub>2</sub> solution was added to the solids to release all formed oligopeptides and the liquid phase was taken for analysis. Control experiments with solution in vials without any catalyst were also carried out.

## 2.3. Analytical methods

All samples were analyzed by a Hewlett-Packard HP-1090M HPLC apparatus using a Shannon Hypersil (ODS 5 μm/200.2.1 mm) column. The mobile phase was a solution of 10 mM sodium hexanesulphonate, adjusted to pH 2.5 by H<sub>3</sub>PO<sub>4</sub>. Before analysis each solution was diluted with mobile phase (1:1). Injection volume was 25 or 20 μl. Mobile phase flow

rate was 0.35 ml/min and column temperature was 40°C. Detection was performed with a diode array detector at 195 nm. Peptides were identified by retention times of authentic reference substances and UV–VIS spectra. Linear oligopeptides and cyclic anhydrides were characterized by the light absorption at 195 nm. On the other hand, the spectra of some other unknown products (probably formed by a decomposition and an oxidation) were identified also by the bands at higher wavelengths. Details of the analytic methods and retention times of analysed compounds have been published elsewhere [19]. The reaction yields were determined as percentage of the reactant converted into the reaction product.

## 3. Results and discussion

### 3.1. Glycine reaction

Gly is the simplest amino acid and in many aspects the most reactive one. Two main products emerging from gly, were detected: gly<sub>2</sub> and diketopiperazine (DKP), the cyclic anhydride of gly<sub>2</sub>. The reaction yields of these products formed with the respective clay catalysts are summarized in Table 1 and were higher than those of blank experiments (about 0.15% of the sum of gly<sub>2</sub> and DKP yields). Whereas the gly<sub>2</sub> yield was always very low (below 1%), DKP yield was mostly higher than that of gly<sub>2</sub>, having reached several percent in some cases (talc,

Table 1  
The formation of gly<sub>2</sub> and DKP from gly on various clays

	Clay									
	K	P	T	M	H	V	Ch	A	S	
gly <sub>2</sub>	0.25 ± 0.02	0.27 ± 0.01	0.46 ± 0.01	0.46 ± 0.04	<b>0.69</b> ± 0.07	0.40 ± 0.10	0.57 ± 0.15	0.66 ± 0.01	<b>0.72</b> ± 0.03	
DKP	0.12 ± 0.03	0.40 ± 0.07	4.61 ± 0.05	2.10 ± 0.23	4.45 ± 0.36	0.21 ± 0.06	0.56 ± 0.03	1.80 ± 0.28	1.68 ± 0.05	
Sum	0.37 ± 0.05	0.67 ± 0.08	<b>5.07</b> ± 0.05	2.56 ± 0.27	<b>5.14</b> ± 0.32	0.61 ± 0.09	1.13 ± 0.13	2.45 ± 0.28	2.40 ± 0.09	

Reaction yields are given in % of gly incorporated in the reaction product.

K—kaolinite, P—pyrophyllite, T—talc, M—montmorillonite, H—hectorite, V—vermiculite, Ch—chlorite, A—attapulgite, S—sepiolite.

hectorite). The reaction mechanism explains the easier formation of DKP: Glycine dimerization proceeds as bimolecular reaction after the activation of one reactant species at a clay particle edge [23,24] (Fig. 2a) and requires sufficient reactant concentration and suitable orientation of reacting entities. Apparently, DKP formation from gly proceeds only via  $\text{gly}_2$ , which is formed from gly and can be easily activated in the same way as gly (Fig. 2b). The subsequent cyclization reaction of activated  $\text{gly}_2$  is much easier from kinetic points of view [25–28] than the dimerization of gly (Fig. 2b). Such intramolecular reaction does not need the reactant concentration, whereas the condensation reaction of two amino acid molecules does. Therefore, cyclic anhydrides are readily formed from amino acids in other reaction systems as well, e.g., on silica and alumina [19,29]. The sum of  $\text{gly}_2$  and DKP yields, therefore, reflects the real amount of gly having taken part in oligomerization, rather than

the  $\text{gly}_2$  yield alone. Hence, neglect of DKP determination in some earlier works investigating clay catalysed gly oligomerization [8,9], has led to underestimation of the catalytic efficiency of clays for peptide bond formation. Consequently, in this work the sum of yields was chosen as measure for the catalytic efficiencies of tested clays for gly dimerization.

The catalytic efficiency of clays varies considerably. The highest yields (about 5%) were achieved on trioctahedral clays, namely hectorite and talc. Twice lower yields resulted with the dioctahedral smectite montmorillonite and with the fibrous clays attapulgite and sepiolite. The lowest catalytic efficiency was observed for kaolinite (Table 1). The interpretation of these results requires a consideration of the mechanisms of amino acid activation on clay surfaces.

There is some theoretical and experimental (spectroscopic) evidence [23,24] that amino acids (oligopeptides) are activated by the same

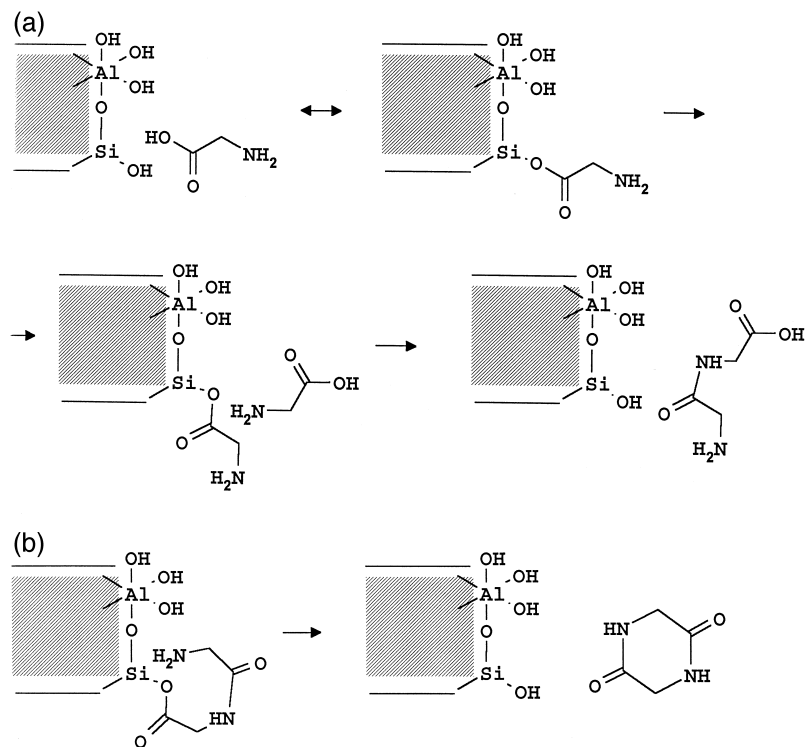


Fig. 2. (a) Scheme showing glycine activation at clay particle edge leading to diglycine formation. (b) Scheme showing the formation of diketopiperazine from diglycine activated at clay particle edge.

way as on a silica surface, i.e., via a condensation (esterification) reaction with Si–OH groups (Fig. 2a,b). A major part of the clay surface (basals) is inactive for the catalysis of peptide bond formation, as amino acids are activated only at clay particle edges [23,24] and possibly at sites of structural defects, which, together, is only a very small fraction of the total surface. The extent of activation depends not only on the reaction step itself, but also on the accessibility of the catalytic sites. The accessibility of these catalytic sites depends on the stability of clay suspensions. For example, smectites as hectorite and montmorillonite are characterized by interlamellar swelling and formation of relatively stable suspensions. The mobility of reactant molecules in such a system would insure their suitable distribution (if the reactant is present in sufficient amount) over the whole active surface of clay. In such a case (hectorite, montmorillonite), a sufficient fraction of clay particle edges is available for catalysis, and the yields are relatively high (Table 1). The yield obtained on the trioctahedral smectite hectorite is higher than that on the dioctahedral montmorillonite, which may be interpreted by different charge distributions on oxygen atoms in octahedral and tetrahedral sheets. Mg–O and Li–O bonds in hectorite octahedral sheets are very polar, more or less ionic, whereas Al–O bonds in montmorillonite are more covalent. Consequently, the octahedral oxygen atoms in hectorite would be more negatively charged, which would facilitate a suitable amino acid zwitterion orientation for

the condensation reaction: positively charged ammonium groups would associate mostly with oxygen atoms in octahedral sheets, free Si–OH groups could react with  $\text{COO}^-$  groups of the amino acid [17]. The same would hold for another trioctahedral clay talc.

Fibrous clays as attapulgite and sepiolite do not swell in water. On the other hand, a significant part of their surface might be suitable for amino acid activation. Similar to zeolites, the channels inside the particles of these minerals are free for the adsorption of water and small molecules as amino acids. Active Si–OH groups are supposed to be present also inside the channels of clay particles, due to imperfect connections of the bands of tetrahedral sheets.

Recently, a study of pH effect on gly oligomerization in solution was reported [30]. A significant increase of  $\text{gly}_2$  yields was observed in heated gly solutions for  $\text{pH} > 7$ . Higher pH decreases the amount of zwitterions in favour of amino acid anions, and since  $\text{COO}^-$  groups of anionic gly are still reactive enough and a higher concentration of nucleophile ( $\text{NH}_2^-$  groups) results, higher yields of  $\text{gly}_2$  are formed. This is in accordance with a previous study investigating the pH effect on the clay catalyzed peptide bond formation, in which an even slight acidification of reactant solutions was found to decrease the peptide bond formation [21]. This pH effect was pointed out to play an important part also in the reaction of amino acids on clay minerals, carbonates, etc. The active sites at the clay particle edges, namely those of Mg-tri-oc-

Table 2  
The formation of DKP,  $\text{gly}_3$  and  $\text{gly}_4$  from  $\text{gly}_2$  on various clays

	Clay								
	K	P	T	M	H	V	Ch	A	S
DKP	2.18 ± 0.14	1.61 ± 0.03	3.50 ± 0.14	3.36 ± 0.20	5.34 ± 0.14	4.65 ± 0.31	3.90 ± 0.01	1.56 ± 0.07	3.39 ± 0.32
$\text{gly}_3$	< 0.05	< 0.05	0.63 ± 0.06	0.33 ± 0.03	1.34 ± 0.12	0.43 ± 0.05	0.42 ± 0.02	0.50 ± 0.01	0.76 ± 0.05
$\text{gly}_4$	0.41 ± 0.06	0.21 ± 0.04	0.85 ± 0.13	0.46 ± 0.01	<b>4.18</b> ± 0.32	1.63 ± 0.05	1.21 ± 0.03	0.39 ± 0.10	1.13 ± 0.05
Sum	0.41 ± 0.06	0.21 ± 0.04	1.48 ± 0.17	0.79 ± 0.03	<b>5.44</b> ± 0.32	2.07 ± 0.08	1.64 ± 0.04	0.89 ± 0.10	1.90 ± 0.09

Reaction yields are given in % of  $\text{gly}_2$  incorporated in the reaction product.

K—kaolinite, P—pyrophyllite, T—talc, M—montmorillonite, H—hectorite, V—vermiculite, Ch—chlorite, A—attapulgite, S—sepiolite. Sum—sum of  $\text{gly}_3$  and  $\text{gly}_4$ .

tahedral sheets may partially hydrolyze [31] and thus enhance the suspension pH. In analogy to gly formation in solution, a series of experiments was performed, where water evaporation was not allowed, thus keeping the volume of the suspensions constant. These samples did not produce any gly<sub>2</sub> and DKP, under otherwise identical conditions. On the other hand, the surface basicity of clays might be important for reactions applying drying/wetting cycles. Removal of the water from hydration shells of the exchangeable cations causes strong polarization effect of these cations on the remaining water molecules. Cation hydrolysis and surface acidification proceeds. In this case basic sites at clay particle edges can play an important buffering role and keep the surface pH suitable for peptide formation reaction.

### 3.2. Diglycine reaction

Heating of gly<sub>2</sub> with clays produces DKP, triglycine (gly<sub>3</sub>) and tetraglycine (gly<sub>4</sub>) (Table 2). Clay catalyzed gly<sub>2</sub> reactions are much more complicated, including various mechanisms of peptide bond formation as well as hydrolysis of reactant and reaction products (Fig. 3). For example, gly<sub>3</sub> can be formed by the hydrolysis of gly<sub>4</sub>, or by the oligomerization of gly (product of hydrolyzed gly<sub>2</sub>) itself. DKP, formed with the relatively highest yields, can directly act in the formation of longer oligopeptides by ring-opening and addition of another component, forming gly<sub>3</sub> or gly<sub>4</sub> in its reactions with gly or gly<sub>2</sub>, respectively (Fig. 3). Investigation of the effects of clay composition or structure on gly<sub>2</sub> oligomerization is, therefore, much more com-

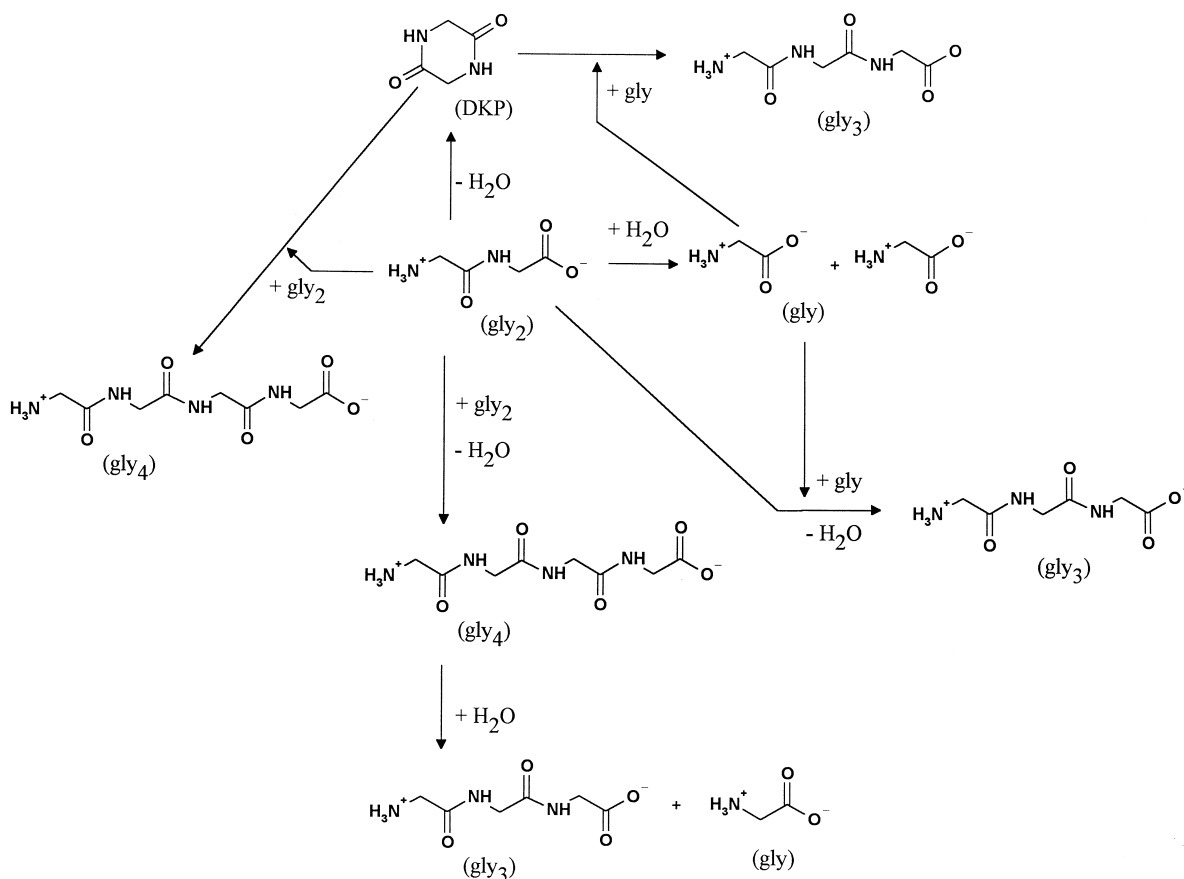


Fig. 3. Assumed main reaction paths in the experiments starting with diglycine.

plicated, taking into account the possibility of various catalytic efficiencies of the clays for the various reaction steps. Nevertheless, there are trends similar to the clays' catalytic efficiency for gly reaction. Hectorite produces the highest yields of both linear oligopeptides and DKP. The yields of linear peptides produced on other clays were at least twice lower. The lowest amounts of gly<sub>3</sub> and gly<sub>4</sub> were produced on kaolinite and pyrophyllite. The low catalytic efficiency of these clays could be explained by the same reasons as discussed for gly reaction.

### 3.3. Reactions including alanine

Experiments starting with ala + gly (gly<sub>2</sub>)/clay suspensions confirmed the significantly lower reactivity of ala, compared to gly. Dialanine was not detected after the reactions on any of the clays investigated.

Ala + gly reaction on hectorite yielded 0.25 ± 0.03% of ala–gly and only trace amounts (< 0.05%) of gly–ala. Ala–gly was also detected on talc. The other clays formed only gly<sub>2</sub> and DKP, any oligopeptides containing ala unit were not found.

Both hectorite and talc are clays containing Mg-trioctahedral sheets in their layered particles. These clays were the most efficient catalysts also for gly oligomerization. In order to find whether basicity of these minerals plays an important role in ala oligomerization with gly, the pH effect was studied in separate series of experiments. Ala + gly solutions of varying pH (4–9) were used in reaction systems without catalyst (as blank) and with montmorillonite and hectorite. If pH played a dominant role in clay catalysed formation of ala–gly, this compound should be found also in the experiment starting with high pH. Phosphate buffers could not have been used, since they are known to catalyse peptide bond formation themselves [32]. Moreover, some preliminary experiments have indicated adsorption of phosphate ions to clay particle edges, resulting in a blockage of these catalytic sites [33,34]. Consequently, the systems

of clays together with phosphate buffers produced much lower yields of gly<sub>2</sub> and DKP than either clays or phosphates alone. Therefore, pH was adjusted by HCl or KOH, respectively. Indeed, higher pH (8–9) ala + gly solutions yield more gly<sub>2</sub> and DKP in blank experiments (about 1% at pH 9), but no formation of dimers containing ala was observed. On the other hand, there was no significant effect of pH on the reaction yields in the experiments with hectorite and montmorillonite. Only a slight decrease of gly<sub>2</sub> and DKP yields was observed in the reaction systems with pH 4. Base catalysed gly oligomerization, recently observed in aqueous solution [30], likely plays a role in peptide bond formation on clays, as already shown in the gly and gly<sub>2</sub> experiments. However, it is not the only mechanism taking place on clays. Higher pH increases the amount of available NH<sub>2</sub>-nucleophilic groups, which enhance the probability of the reaction with another amino acid molecule activated at a clay particle edge.

The experiments of ala + gly<sub>2</sub> reactions proved the relatively higher reactivity of gly<sub>2</sub>. Ala–gly–gly was formed as a main product, which is in accordance with previously published works [19,22]. Only traces of gly–gly–ala were detected, likely produced by ring-opening of DKP and ala addition [19–21,32,35]. Hectorite produced the highest yields: 0.43% ala–gly–gly and 0.18% gly–gly–ala. The yields formed by other clays are lower at least by a factor of two and decreased in the order: hectorite > talc = sepiolite > montmorillonite = attalugite. Chlorite and vermiculite formed only traces of ala–gly–gly, and none of the tripeptides was detected in the reactions with kaolinite and pyrophyllite. Hence the order of catalytic efficiencies of clays is very similar to that observed in gly and gly<sub>2</sub> experiments.

## 4. Conclusion

Testing various clays in model reactions for peptide bond formation confirmed the effect of

clay structure and composition on the efficiency of these catalysts. Trioctahedral clays of rather basic surface, such as hectorite and talc, are the most efficient ones for this purpose. Besides mineral surface basicity, also other parameters can be supposed to play a significant role, such as structural effects related to the activation of reactant molecules and the accessibility of the catalytic sites.

## Acknowledgements

Thanks are due to the Austrian Federal Ministry for Science and Transport for financial support within the bilateral cooperation with Slovakia (GZ.45.423/1-IV/B/3a/97) the Slovak Grant Agency for Science VEGA (grant No. 2/4042/98) and the Austrian Academy of Sciences for supporting a research stay of Dr. Juraj Bujdák at the University of Innsbruck within the Agreement of Exchange between National Academies of Science.

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