Comparative Study on the *N*-acylase Activity of Mammalian Kidney Acetone Powders (KAP's)

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Abstract. The *N*-acylase activity and enantioselectivity of mammalian kidney acetone powders (KAP's) or their enzyme extract was demonstrated on (*rac*)-*N*-acetylmethionine; used as reference substrate. It was showed hydrolysis exclusively on the (*S*)-enantiomer. The biocatalyzed reaction allowed us, to categorize the KAPs regarding the animal source, sheep, pig, calf, bovine, dog and guinea pig as fast biocatalysts reaching equilibrium in around 4 to 5 h; and rat, mouse and hamster were slower biocatalysts, since they did it in around 24 h. In most of the reactions the kidney crude preparations gave a better conversion than the enzyme extract, this fact demonstrated that the longer stirring during the reaction in an aqueous medium, allowed a greater dissolution of the enzyme. These readily available and inexpensive crude biocatalysts have a great potential application in organic synthesis.
Key words: Kidney Acetone Poder, Acylase, Enzymatic Resolution, Amino Acids, Deacylation.

Resumen. La enantioselectividad y actividad *N*-acilasa de los polvos acetónicos de riñón de mamíferos (KAP's) o su extracto enzimático fue demostrada sobre *N*-acetilmetionina racémica, la cual fue usada como substrato de referencia. La hidrólisis mostró preferencia exclusiva por el enantiómero-*S*. La reacción biocatalizada también permitió diferenciar los KAP's de acuerdo a la fuente animal, borrego, cerdo, ternera, res, perro y cuyo alcanzaron el equilibrio en aproximadamente 4 a 5 h; y rata, ratón y hámster lo hicieron en más de 24 h. En la mayoría de las reacciones, las preparaciones crudas de riñón dieron mejor conversión que el extracto enzimático, este hecho demostró que la agitación prolongada durante la reacción, permitió mayor disolución de la enzima. Estos biocatalizadores crudos, fácilmente disponibles y de bajo costo, tienen un gran potencial de aplicación en la síntesis orgánica.
Palabras clave: Polvo acetónico de riñón, acilasa, resolución enzimática, aminoácidos, desacetilación.

Introduction

Modern biocatalysis searchs for development of novel, precise, environmental and cheaper tools to improve a wide range of processes, which will reduce energy and raw material consumption, and to generate less wastes and toxic side-products. The principal drawbacks hindering a broader applicability of enzymes are the high production cost and the low yields [1]. In recent years, the use of enzymes has gained wider acceptance, thanks to enzymes from plant, animal and microbial sources show several advantages over conventional chemical transformations; especially regarding regioselectivity and steroselectivity. The enzymatic chemical applications most studied are those involving hydrolases, mainly lipases and esterases.

The aminoacylases (*N*-acyl amino acid amidohydrolases, EC 3.5.1.x) are hydrolases belonging to a family of enzymes that catalyze stereospecifically the hydrolysis of *N*-acetylated aminoacids. Kidney acylase was discovered as early as 1881, originally called "histozyme" [2]. In 1922 Smorodinzew showed that this enzyme was able to hydrolyze a wide variety of acyl-aminoacids and then introduced the *acylase* concept [3]. The term acylase was first coined in 1952 to designate the enzyme, contained in a crude preparation of hog kidney, responsible for the asymmetric hydrolysis of the *N*-acetyl group in acetylated natural aminoacids, over the *L*-enantiomers. A more refined study led to the discovery of two kind of acylases, the first one called Acylase I (Acy I) which acts over most of the aa's except aspartic acid, and the second one, Acylase II more active over the aspartic acid [4].

Aminoacylase I (Acy-I, EC 3.5.1.14) is found in many mammalian tissues, with the highest activity occurring in kidney [5]. The enzyme hydrolyzes a variety of *N*-acylated amino acids; however, the physiological role and the exact cellular localization of Acy-I is still a matter of debate. The comparison of Acy-I activities in kidney and liver homogenates of 11 mammalian species showed that the enzyme is most abundant in true herbivores such as sheep and cattle as well as in omnivores, while activities were lower in both rodents and cats. Acy-I activity was not detected in dog liver of five different breeds [5]. Acylase I showed to be present, by immunoassay in rat kidney, lung, liver, brain, stomach, intestines, adrenal glands, pancreas and testis [6].

Based on the results and literature data, it was proposed that Acy-I catalyzes the synthesis (rather than hydrolysis) of hippurate, which is formed as a detoxification product of aromatic compounds.

Regarding the application of this enzyme in organic chemistry, the acylase from *Aspergillus* spp was used in a key step for the preparation of the pheromone of the queen of the oriental hornet, *Vespa orientalis* [7]. The same acylase in conjunction with porcine acylase has been used for resolution of several unnatural and rare occurring aminoacids [8].

Other application of acylases is the preparation of (*S*)-α-amino-(2-chlorophenyl) acetic acid, at multigram scale, by the enantioselective hydrolysis of the corresponding *N*-phenylacetyl derivative using immobilized penicillin G acylase [9]. The (*rac*)-dehydroleucine was prepared and resolved by porcine

kidney acylase during the preparation of alloviroidin, a natural product isolated from *Amanita suballiace* [10].

On the other hand, acylase I from *Aspergillus melleus* catalyzed the transesterification and acylation of secondary alcohols [11]. It is interesting to note that Acylase I from pig kidney showed to be the best among 57 commercial acylases for the esterification of aminoacids [12].

Acylases are present in numerous mammalian tissues with the highest activity occurring in kidney and being less important in liver, with approximately 25% of kidney content [5]. An arylamine acyltransferase has been isolated from pigeon, chicken and duck livers (as acetone powders) with equivalent content of the enzyme; with the pigeon acylase having a sevenfold higher activity, but chicken liver preparation is 40 times cheaper [13].
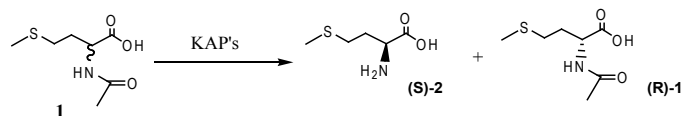
The aim of this work was to determine the acylase activity of several crude preparations of mammalian kidney acetone powders (KAP's), using (*rac*)-*N*-acetylmethionine as a model substrate.

Results and Discussion

From the work on the Liver Acetone Powders (LAPs) [14, 15, 16], we were encouraged to explore the use of kidney acetone powders (KAPs) readily available, easy to handle and cheap as biocatalyst in organic chemistry. We found that pig kidney acylase (acylase I) has been widely used [4, 17], even the reaction mechanism has been studied [18], but there are not studies on other mammalian kidney acylases. Therefore, the acetone powder of sheep, pig, calf, bovine, cat, dog, guinea pig, mouse, rat and hamster kidneys were prepared following the general procedure applied for the liver acetone powders (LAPs) [19]; to be used as a crude biocatalysts.

The hydrolysis of (*rac*)-*N*-acetylmethionine (1) was selected as a model for the evaluation and comparison of the acylase activity of the home-made KAPs (Scheme 1), even though the reaction do not go to completion but to an equilibrium, as reported by swine (sheep) kidney acylase [20]. It is worth to mention that this amino acid is commonly used to evaluate the acylase activity of commercial acylases.

In order to evaluate the acylase activity in selected KAPs, we developed an analytical method to carry out the monitoring progress of the reaction; the typical colorimetric (ninhydrin) procedure [21] was difficult to reproduce even for the calibration curve, this method resulted to be too sensitive to the time and temperature of the reaction, which led to great variations in the results. In consequence, we decided to use HPLC, since it is an easier to handle and more reproducible method.

The chromatographic separation presented enough enantiomeric resolution to reagents (1) and products (2), to allow the direct analysis of the crude reaction mixture for conversion and enantioselectivity determination. The enantiomeric assignment was done by comparison of the sign of the optical rotation of reaction product [(S)-methionine, (*S*)-2)] with the reported data [4], and the elution order (retention times in HPLC analysis) [22, 23]. The calibration curve for the (*S*)-enantiomer (Figure 1), allowed the quantitative determination of the product from the enzymatic hydrolysis reactions.

Once the analytical method for monitoring the reaction progress was established, the sample preparation from the biocatalyzed reaction was addressed. In an attempt to stop the reaction, we first tried removing the solid biocatalyst by filtration through syringe filters (45 microns) followed by storing the samples in a refrigerator (4 °C) for further analysis. The HPLC chromatograms of all samples, regardless of the reaction time, showed practically the same conversion for each KAP. This fact led us to consider the possibility of the enzyme got in solution, and for that reason the reactions continued, even during refrigeration.

These results showed that the removal of the solid KAP was not enough to stop the reaction; that forced us to look for another method for sample preparation before the HPLC analysis for monitoring of the reaction. We tried heating the reaction mixture at 90-95°C for 3 min, followed by centrifugation (13000 rpm/ 5 min) and HPLC analysis; but once again the results showed that the reaction reached the equilibrium even for aliquots taken after 5 min of reaction. The results demonstrated that the enzyme was not completely deactivated during that heating. Then we tried by increasing the temperature to 110-115°C to achieve complete enzyme deactivation, but some evaporation took place making the quantification results non reproducible.

Finally we found that the best procedure for deactivating the enzyme, and therefore to stop the enzymatic reaction, was the addition of a 10% trichloroacetic acid (TCAA) solution, letting the mixture sit for 2 min followed by centrifugation and HPLC analysis; this procedure allowed us to store the samples for further HPLC analysis.

With the idea that the enzyme was dissolving, two types of experiments were developed, in the first one the KAP was
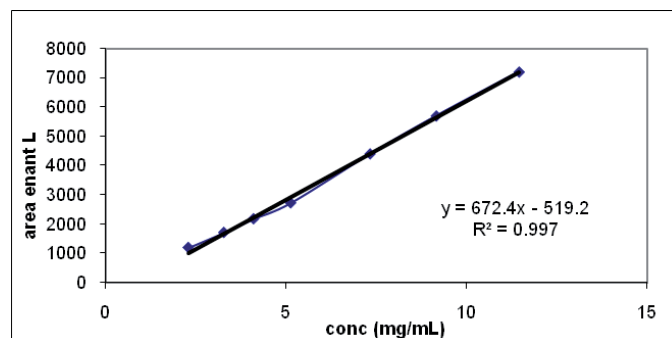

Scheme 1.

Fig. 1. Calibration curve for (*S*)-methionine.

The calibration curve shows: $y = 672.4x - 519.2$, $R^2 = 0.997$

suspended in a buffer solution and stirred for 30 min to allow the enzyme to dissolve followed by removing the solid KAP by centrifugation, the supernatant was used for the reaction. In the second experiment the KAP was kept along the reaction.

Figure 2 shows the time in which each KAP reaction reached equilibrium; it is worth to mention that all reactions were monitored for 26 h. As it can be seen the KAPs can be classified in two big groups, one in which the equilibrium was reached in around 4-5 h, for sheep, pig, calf, bovine, dog and guinea pig KAPs, being all of them faster than the second group consisting of rat, mouse and hamster KAPs, which reached the equilibrium in more than 24 h. Faster reactions were observed using the whole KAP rather than experiments with soluble fraction of the enzymes; this fact may be due to the time used in the experimental procedure for dissolving (30 min) of the enzyme allowed just partial solution, in contrast longer stirring of the KAP in the aqueous reaction medium, during the whole reaction increased the dissolution of the enzyme. Another factor could be the different texture of each KAP that may lead to differential dissolution of the enzyme from the KAP matrix.

The results in Figure 3 show the conversion percentages, after 24 h of reaction, based on the 50% maximum (in this case considered as 100% conversion) for the complete resolution. From the graph it is obvious that all the reactions reached an equilibrium state, but none of them got a complete resolution under the reaction conditions tested. In the cases of calf, bovine, cat and rat KAPs, the reactions with the soluble fraction of the enzymes conducted to minor conversion. As mentioned before this fact may be due to the time used for the dissolution of the enzyme. In pig, dog, guinea pig, mouse and hamster KAPs cases, the extent of the hydrolysis reaction was similar in both kinds of experiments, however for sheep the conversion was greater for the soluble enzyme.

It is remarkable that in all cases only the *S*-enantiomer was hydrolyzed, as demonstrated by the lack of the *R*-enantiomer in the HPLC chromatograms, even after 24 h reaction; so the reaction was highly enantioselective, with ee's greater than 99% in all cases. The enantiomer assignation was done by comparison with sign of optical rotation reported in literature [4].
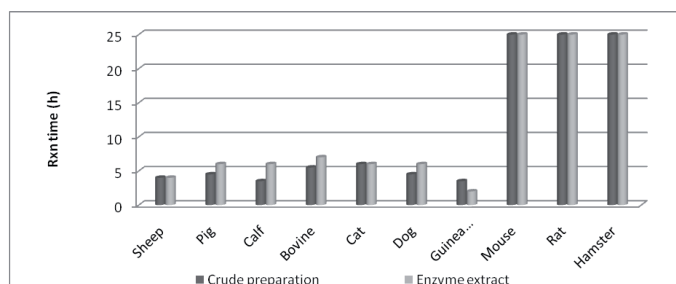

Fig. 2. Comparison of time to reach the equilibrium from the enzymatic hydrolysis of *(rac)-N*-acetylmethionine (1), using crude KAPs (dark columns), and enzyme extract (light columns). The reaction times (Y axis) correspond to time for the flattening of the monitoring curve during the resolution process.
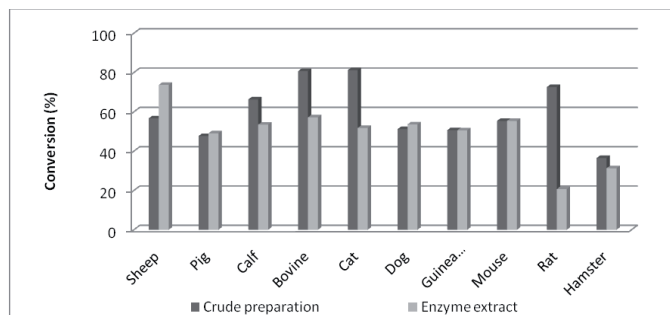

Fig. 3. Comparative conversion percentages from the enzymatic hydrolysis of *(rac)-N*-acetylmethionine (1), after 24 h of reaction, using crude KAPs (dark columns), and enzymatic extract (light columns). The conversion percentages were calculated based on the maximum of 50% conversion for a theoretical resolution process.

Experimental

Chemicals and materials. All reagents and solvents were commercially available and used without further purification. Bovine, sheep, calf and hog (pig) kidneys were obtained from recently slaughtered animals from a nearby slaughterhouse. Dog and cat kidneys were supplied by the local official dog control center and rat, mouse, guinea pig and hamster kidneys from the animal breeding facility of our University.

Analytical method. (*rac*)-methionine and *(rac)-N*-acetylmethionine were analyzed by HPLC using a Chirobiotic-T column (from Astec) [22] with triethylamine (TEA)/acetic acid buffer (4 mM, pH 4.4)-methanol (90:10) as eluent, with a flow of 0.8 mL/min and detection at 220 nm. Retention times: (*S*)-methionine, 4.7 min; (*R*)-methionine, 5.2 min; (*S*)-*N*-acetylmethionine, 3.9 min; (*R*)-*N*-acetylmethionine, 9.9 min.

Calibration curve. 114.6 mg of (*rac*)-methionine were dissolved in 10 mL of 0.1M phosphate buffer (pH 7.5) (prepared in HPLC grade water). From this solution several dilutions were done in order to get the following concentrations: 11.46, 9.17, 7.33, 5.13, 4.11, 3.28, 2.3, mg/mL. To a 20 μL of each solution, 20 μL of 10% trichloroacetic acid (TCAA) was added, shaked, let stand for 2 min and subjected to HPLC analysis. The results were plotted to get the calibration curve for the *L*-enantiomer.

General procedure for Kidney Acetone Powder (KAP) preparation. Fresh sheep kidney (142 g) was blended in 420 mL of chilled acetone using a household blender and vacuum filtered through a Büchner funnel. The resulting cake was treated in the same manner twice. The solid residue was air dried under the hood and sifted on a 20 mesh sieve. The procedure yielded 22.4 g of dry powder, which was kept at 0-4° C. All the KAP's were prepared in a similar manner.

General procedure for the enzymatic hydrolysis of (rac)-N-acetylmethionine, using crude KAP's. A *N*-Acetylmethionine buffer solution (11.4 mg/mL) with 0.6 mL of 2mM $CoCl_2$ solution (all at pH 7.5), was pre-heated at 36-37° C for 15 min, then the KAP was added (1:1, w/w) and the reaction mixture magnetically stirred. Aliquots were taken to monitor the progress of the reaction up to 24 h, according to each KAP. The

aliquots of 0.1 mL of reaction mixture were mixed with 0.1 mL of 10% TCAA into an Eppendorf vial, the mixture was shaken and let stand for 5 min, then centrifuged (13000 rpm/3 min), and transferred to an HPLC vial, stored in refrigeration, for the HPLC analysis.

General procedure for the enzymatic hydrolysis of (rac)-N-acetylmethionine, enzyme extract. 19-20 mg of each KAP was suspended in 1.0 mL of phosphate buffer (pH 7.0) and stirred at 37° C for 30 min; then the mixture was centrifuged (13000 rpm for 3.5 min). The supernatant was added to 9.0 mL of a *N*-acetylmethionine buffer solution (11.4 mg/mL) with 0.6 mL of 2mM $CoCl_2$ solution (all at pH 7.5), and magnetically stirred; aliquots were taken to monitor the progress of the reaction for up to 24 h, according to each KAP. The aliquots of 0.1 mL of reaction mixture were mixed with 0.1 mL of 10% TCAA into an Eppendorf vial, the mixture was shaken and let stand for 5 min, centrifuged (13000 rpm/3 min), then transferred to an HPLC vial, stored in refrigeration, for HPLC analysis.

Conclusion

The *N*-acylase activity and enantioselectivity of several mammalian sources was demonstrated using their KAP's or the enzyme extract in phosphate buffer (pH 7.5). These biocatalysts showed high enantioselectivity on (*rac*)-*N*-acetylmethionine, used as a model substrate, the exclusive preference for the hydrolysis of the *S*-enantiomer is relevant, and prompted us to continue the study of this methodology. Further work will be focused on new substrates and the application in the synthesis of commercial compounds. These readily available and not expensive crude biocatalysts have great potential in organic synthesis.

Acknowledgments

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