Materials and Methods

A. Native PAGE and SDS–PAGE

To confirm the purity of the enzyme, discontinuous native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)–PAGE were performed according to the previously described methods of Hames (1998) and Laemmli (1970), respectively. Silver staining of the gels was achieved according to the method of Blum *et al.*, as described earlier (Blum et al., 1987). For activity staining of the native gels, Juul's method was employed (Juul, 1968).

B. Enzyme Assays

Enzyme activity was measured on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer according to the method of Ellman *et al.* (1961) as described earlier. Here, the substrate butyrylthiocholine (BTCh) was hydrolyzed by BChE, and thiocholine was released. The free thiocholine then reacted with 5,5′-dithio-2-bis-nitrobenzoate (DTNB) and produced the thionitrobenzoate (TNB) anion which absorbed light at 412 nm. For the physicochemical characterization of horse serum BChE, optimum pH, optimum temperature, energy of activation (E_a) and temperature coefficient (Q_{10}) were determined accordingly (Segel, 1975).

C. Determination of Optimum pH

BChE activity was measured by using MOPS buffer (50, 100, 150 and 200 mM) prepared at seven different pH values to better understand the effect of pH on reaction rate. The pH values varied between 6.5 and 9.5. Measurements were carried out according to Ellman's method (Ellman *et al.*, 1961). The reaction mixture consisted of 250 µl of MOPS buffer, 10 µl of 50 mM BTCh, 50 µl of 2.5 mM DTNB, 165 µl of dH_2O, 25 µl of the BChE enzyme and the reaction was followed at 412 nm for 20 s. The activity at each pH value and buffer concentration was tested in triplicates at 37 °C. To eliminate the buffer effect all values were

extrapolated to zero buffer concentration (Landqvist, 1955). Average enzyme activity was calculated for each pH value, and the "specific activity vs. pH" plot was depicted.

D. Determination of Optimum Temperature

BChE activity was measured at different temperature points in to better understand the effect of temperature on reaction rate. The measurements were carried out according to Ellman's method (Ellman *et al.*, 1961). Reaction was followed for 20 s at 412 nm. The reaction mixture consisted of 250 µl of 200 mM MOPS at pH 8.5, 10 µl of 50 mM BTCh, 50 µl of 2.5 mM DTNB, 165 µl of dH_2O, and 25 µl of BChE. In each assay, the reaction medium was heated to different temperatures (20 °C, 25 °C, 30 °C, 35 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C), and the resulting activity at each temperature was measured in triplicates. Average enzyme activity was calculated for each temperature point and the "specific activity vs. temperature" plot was depicted. Using the same data, the Arrhenius plot was depicted for the calculation of energy of activation (E_a) and temperature coefficient (Q_{10}).

Results

Characterization of BChE

Native PAGE and SDS–PAGE were carried out in order to validate the purity and authenticity of the enzyme and also to determine the relative molecular mass of its different oligomeric states. Native polyacrylamide gels were subjected to Coomassie Brilliant Blue R-250 staining (not shown), silver staining and activity staining for comparison purposes. On all gels, two protein bands running close together were observed (Figure S1). We propose that this is because horse serum BChE has eight *N*-linked carbohydrates per subunit (Moorad *et al.*, 1999). The *N*-linked carbohydrates on BChE generally carry two sialic acid moieties; therefore, BChE has several negative charges on it (sixteen per subunit), which may affect the migratory behavior of the protein. This was confirmed by the presence of an overlapping migration pattern on the silver- and activity-stained gels. On the SDS–polyacrylamide gel, however, instead of having a single band corresponding to the BChE monomer, three distinct bands were observed: one for the tetramer (M_r: 380 kDa), one for the dimer (M_r: 190 kDa), and one for the monomer (M_r: 95 kDa) (Figure S2). Horse serum BChE has three intrachain and one interchain disulfide bonds per subunit (Teng *et al.*, 1976; Lockridge *et al.*, 1987). Seemingly, 2-mercaptoethanol at a concentration of 20 mM is ineffective in reducing all the disulfide bonds present. This is in line with studies of other groups who used stronger thiol-reducing agents at even higher concentrations (such as 50 mM dithiotreitol) (Biberoglu *et al.*, 2012). Still, the relative molecular mass of the tetrameric form is in good correlation with the values reported in the relevant scientific literature (Cokugras, 2003; Biberoglu *et al.*, 2012). The physicochemical parameters optimum pH, optimum temperature, E_a and Q_{10} were calculated to be 8.1, 39°C, 1,526 cal mol^{-1} and 1.16, respectively (Figures S3 and S4). Our findings are highly consistent with those of others, including Lee and Harpst (1971).

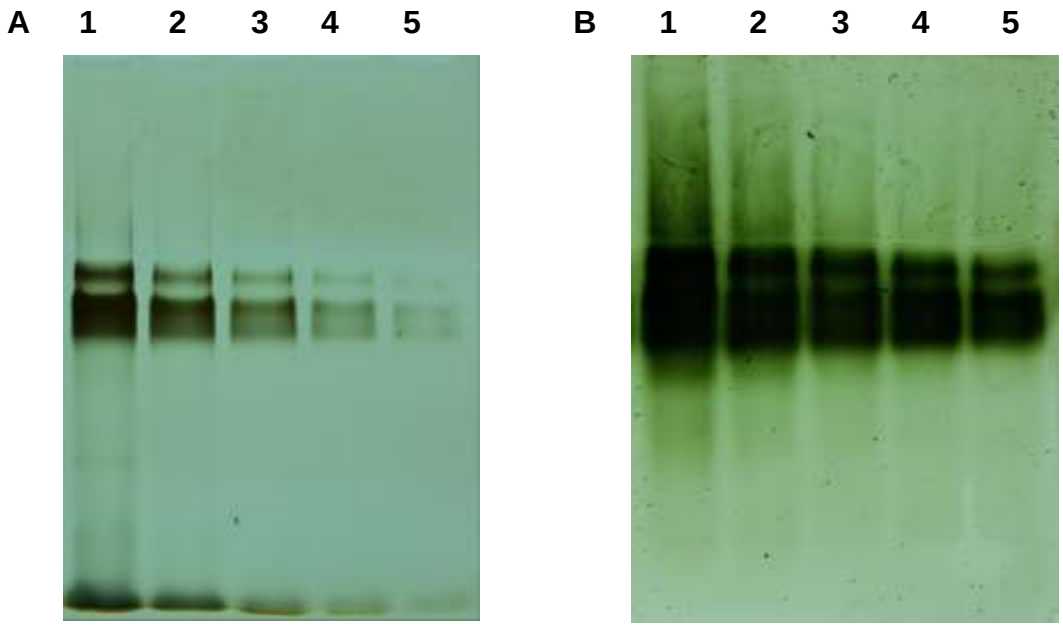

Fig. S1 Visualization of BChE on discontinious native-PAGE by silver (A) and activity (B) stainings. Separating and stacking gels were casted in the concentration of 6% and 4%, respectively. BChE concentration in each lane was as follows: Lane 1, 1.25 µg; lane 2, 0.5 µg; lane 3, 0.25 µg; lane 4, 0.125 µg and lane 5, 0,0625 µg.

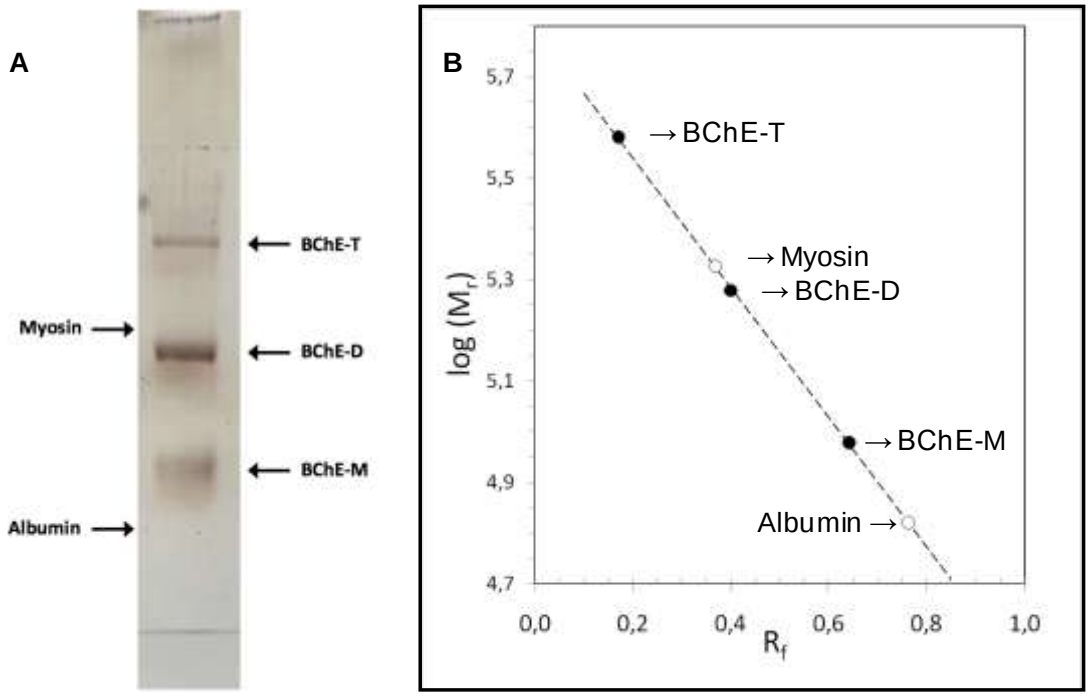

Fig. S2 A. Visualization of BChE on discontinious SDS-PAGE by silver staining. Separating and stacking gels were casted in the concentration of 10% and 4%, respectively. Concentration of BChE was 5 µg. Molecular weights of the myosin and albumin were 212 and 66 kDa, respectively. BChE-T (Butyrylcholinesterase tetramer), BChE-D (Butyrylcholinesterase dimer), BChE-M (Butyrylcholinesterase monomer). Figure 4.4.B. Log (M_r) vs. R_f plot.

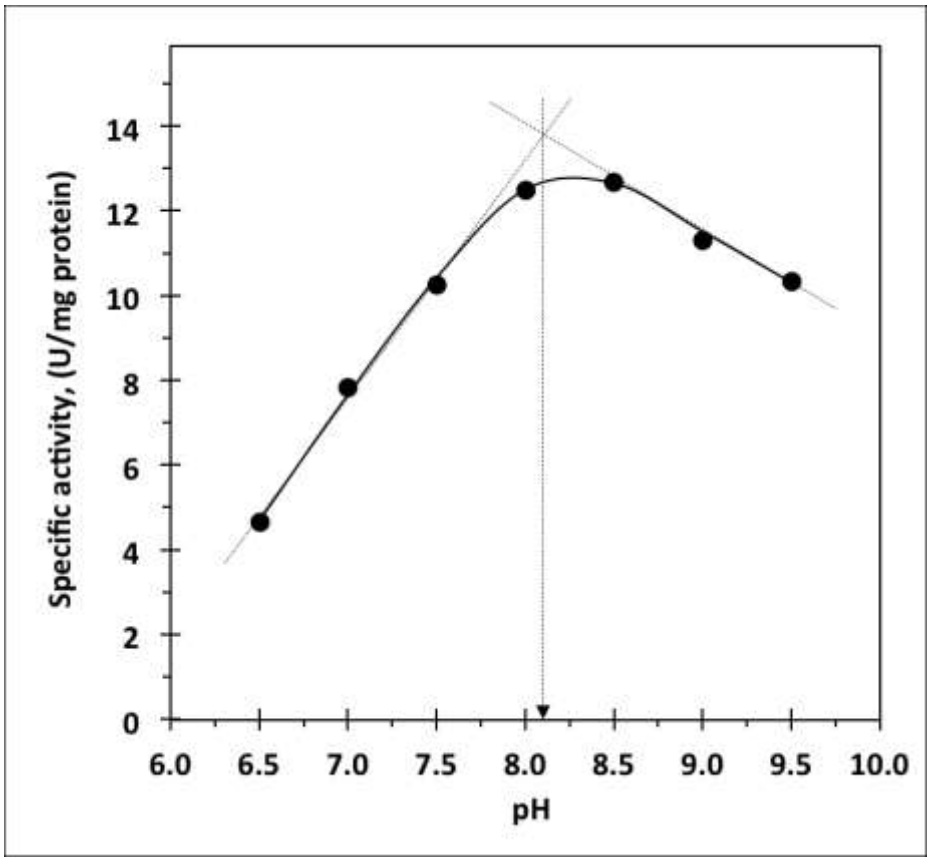

Fig. S3 Determination of the optimum pH. Specific activity (U/mg protein) vs. pH plot

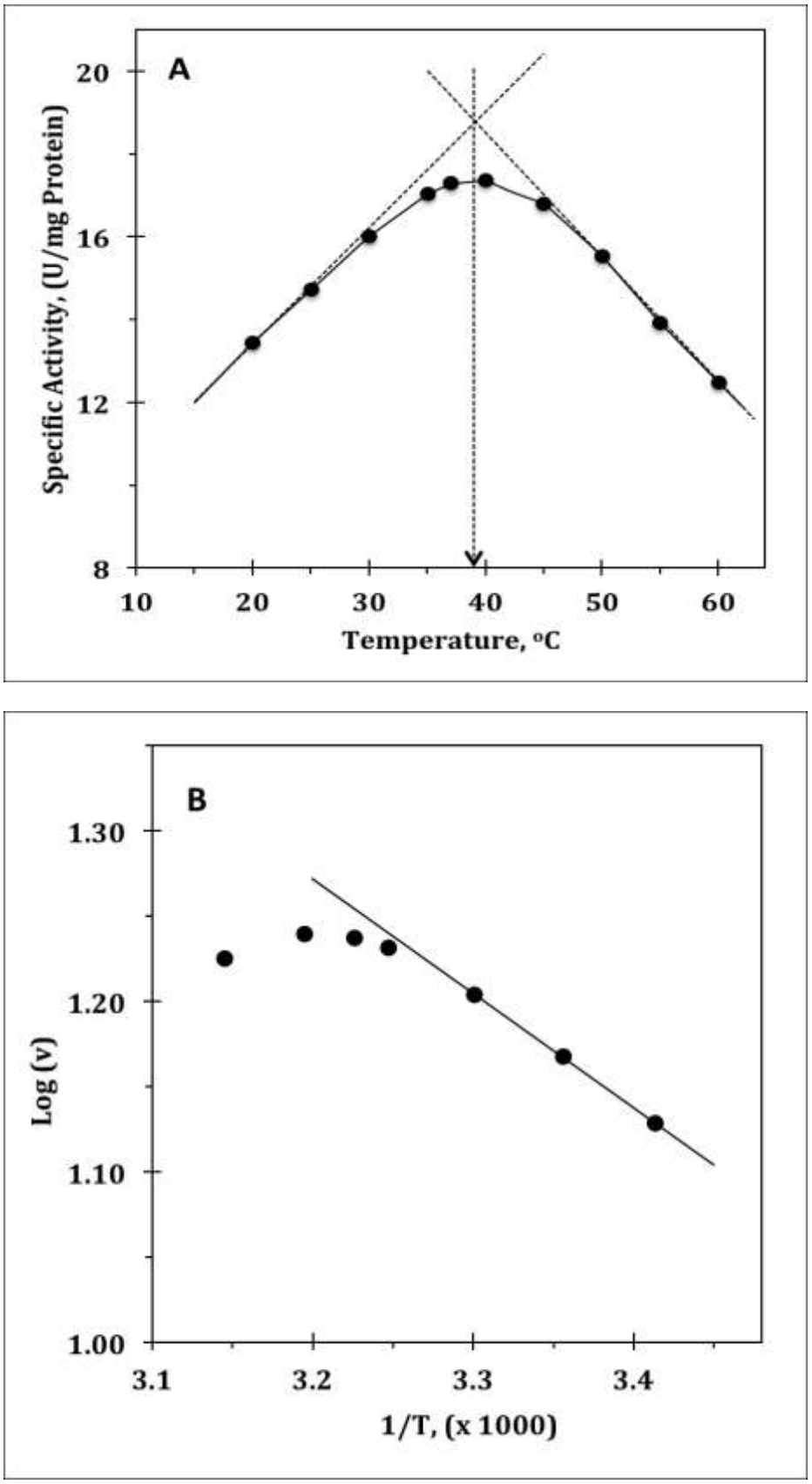

Fig. S4 Determination of the optimum temperature. A. Specific activity vs. temperature plot. B. Log (V) vs. 1/T (Kelvin) plot.

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